National Chung Hsing University

Department of Animal Science

Doctoral Dissertation

Isolation, Identification and Selection of Lactic Acid Bacteria Cultures from Mum (Thai Fermented Sausages) and Their Applications

泰式發酵香腸(Mum)中乳酸菌之分離、鑑定、篩選與應用

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泰式發酵香腸(Mum)中乳酸菌之分離、鑑定、篩選與應用

摘要

Mum，為一種傳統的泰式發酵香腸，普遍生產並消費於泰國東北部地區，但在生產過程中，卻少有科學化和技術被應用於其中。傳統上，mum 香腸是利用天然乳酸菌，因此也易導致產品品質的不穩定。製造商常依生產技術和經驗而並非科技以維持產品質。所以影響產品的最終特性與品質主要來自原物料品質及生產技術。因此，本研究的目的是藉由一種新的方法以改善 mum 香腸之品質及保存期限並利用乳酸菌菌酥的接種以建立一個更穩定並可控制的加工程序。

在試驗一中，兩種不同方法用於生產新鮮 mum 香腸，方法一：香腸於 30℃ 下進行發酵和儲存 14 天;方法二：香腸在 30℃下發酵三天，再以真空包裝存儲於 4℃，直到第 28 天。於過程中取樣測定其物理特性、微生物，質地，感官評品。結果顯示，方法一的樣本之脫水現象較方法二之樣品為明顯並導致較低的水分含量和水活性。在第 3 天時，這兩方法之樣品其 pH 值均有顯著下降但乳酸則有顯著增加。總生菌數及乳酸菌數在發酵過程中，菌數先是迅速升高，然後下降，而腸內桿菌則是持續下降。方法一之樣品則因脫水會造成質地堅硬和不可接受的感官品質。綜上所述，方法二之樣品於三天的發酵後使用真空包裝並儲存在 4°C 下，更能生產較佳品質且保存期限較長的 mum 香腸。

在試驗二中，從試驗一中之 2 種方法製造的 mum 香腸進行乳酸菌的分離，再將分離出的乳酸菌(LAB)其 16S rDNA 利用聚合酶鏈反應擴增，並用限制內切酶進行消化。結果顯示，*Dde* I 最具有辨別能力。乳酸菌於更進一步分類後共計有 *Lactobacillus sakei, Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Lactobacillus brevis*, *Lactobacillus fermentum*, *Pediococcus pentosaceus*, 和 *Lactococcus lactis*。 在這兩種製造方法中 *L. sakei* 和 *L. plantarum* 在發酵和儲存過程中是為優勢菌種。而方法二中，*Ln. mesenteroides* 的比例於儲存熟成期間很明顯增加且類似於 *L. sakei* 是為次優勢菌。樣品中的乳酸菌的鑑定將有助

於選擇適當的菌酰以進一步使用。

在試驗三中，添加混合發酵菌酰（*L. plantarum*，*L. sakei* 和 *Lc. lactis*）及亞硝酸鈉（125 ppm）是可提高 mum 香腸之產品品質和安全性。結果顯示不僅可顯著降低 mum 香腸之 pH 值及抑制腸內桿菌生長，亦可抑制 TBARS 及 VBN 的積累；同時有減少亞硝酸鹽的殘存量，及提高紅色值的效果。另外，使用混合發酵菌酰與亞硝酸鹽所生產之 mum 香腸，其各項感官評品尤其是風味，顏色和總接受性均有最高的得分。

綜上所述，利用真空包裝、混合發酵菌酰和亞硝酸鹽的組合，證實能提高 mum 香腸之品質及安全性並延長其保存期。

關鍵字: 泰式香腸，乳酸菌

Isolation, Identification and Selection of Lactic Acid Bacteria Cultures from Mum (Thai Fermented Sausages) and Their Applications

ABSTRACT

Mum, which is a traditional Thai fermented sausage, is commonly produced and consumed in the north-eastern region of Thailand, and yet little scientific and technology information is applied in production. Traditionally, mum sausage is produced by utilizing natural occurring lactic acid bacteria (LAB), and it may cause the products with inconsistent qualities. Their production depends on the skill and experience of the meat manufacturers rather than technology. Hence, the specific characteristics and qualities of final products are mainly influenced by the raw materials source, the manufacturing techniques and the agro-ecosystem of the production area. Thus, the aim of this study was to improve the qualities and the shelf-life of mum sausages by a new method and also to develop desirable LAB starters in order to establish a more controllable processing.

In experiment I, freshly-manufactured mum sausages were produced by two processing methods (process I: fermented and stored at ~30°C for 14 days; process II: fermented at ~30°C for three days, vacuum-packaged and stored at 4°C until day 28). Physiochemical, microbial, textural, and sensory properties of the sausages were analyzed. The results showed that dehydration was more extensive in process I samples, and resulted in lower moisture content and water activity than process II samples. Significant decreases in pH values and increases in lactic acid were observed in both samples at day 3. The total microflora and lactic acid bacteria counts increased rapidly during the fermentation and then decreased as *Enterobacteriaceae* counts decreased. Extensive dehydration resulted in tough textures and unacceptable sensory qualities for the process I samples. In conclusion, three days of fermentation, with vacuum-packaging, ripening and storage at 4°C, were possibly to produce mum sausages with better qualities and more extended shelf life.

In experiment II, LAB were isolated from the sausages during manufacturing by two methods (method I: after stuffing, sausages were fermented and stored at ~30°C for 14

days; method II: after stuffing, sausages were fermented at ~30°C for three days, then were vacuum-packaged and stored at 4°C until day 28). The 16S rDNA of LAB isolate was amplified by polymerase chain reactions and digested with restriction enzymes. The results showed that *Dde* I presented the highest discrimination capacity. LAB were classified and further identified as *Lactobacillus sakei, Lactobacillus plantarum, Leuconostoc mesenteroides, Lactobacillus brevis, Lactobacillus fermentum, Pediococcus pentosaceus*, and *Lactococcus lactis*. During fermentation and storage, *L. sakei* and *L. plantarum* were dominant in both methods. For method II, the proportion of *Ln. mesenteroides* increased remarkably during post-ripening storage and became predominant similar to *L. sakei.* The identification of LAB in samples helps to select appropriate microorganisms as candidate starter cultures for future use.

In experiment III, the addition of selected mixed starter culture (*L. plantarum, L. sakei,* and *Lc. lactis*) with $NaNO_2$ (125 ppm) had a great potential to improve quality and safety of mum sausages, which showed a significant decrease in pH values, inhibiting the growth of *Enterobacteriaceae*, and suppressing the accumulation of TBARS and VBN. Also, it showed an ability to reduce nitrite content and revealed a higher redness values. Moreover, sausages produced with mixed starter culture and nitrite had the highest scores in most sensory attributes especially in flavor, color, and overall acceptance.

Thus, the combination of several hurdles including vacuum-packaging, mixed starter culture and additive in the manufacture of mum product evidenced that can improve its qualities, safety and extend the shelf-life.

Keywords: Thai fermented sausage, Lactic acid bacteria

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CHAPTER 1

Introduction

1. Rationale and Background

Fermentation of meat, a worldwide and oldest technique in food preservation, was originally used to store food for longer times (Hutkins, 2006). This principle is employed not only to ensure microbiological safety, but also to enhance the flavor and nutritional quality of product. The technique allows a low energy conservation of meat and yields a high quality product. During fermentation, complicated microbial, biochemical, and physical reactions take place resulting in a significant change of sensorial characteristics (Demeyer and Toldra, 2004). According to the issue of Zeuthen (1995), the shelf-life was based on natural lactic acid bacteria (LAB), traces of nitrate and high amount of salt. LAB have a long history as generally recognized as safe (GRAS) organisms, due to the lactic acid and other organic acids produced by these bacteria to act as a natural preservative as well as flavor enhancers. LAB are increasing acceptance for use as probiotics which aid in stimulating immune response, preventing infection by enteropathogenic bacteria and preventing diarrhea (Reid, 1999).

After World War II, the need for fermented meat products increased and the use of starters in food fermentations became a mean to increase processing speed and product consistency. Starters were used to improve the sensory characteristics and microbiological quality and to shorten the fermentation time of fermented foods (Visessanguan *et al*., 2006). Researchers have begun to develop starter cultures for meat products in order to ensure a consistent quality for the fermentation process (Caplice and Fitzgerald, 1999), due to the important role of LAB in food fermentation, the isolation and identification of these microorganisms from natural sources are of great scientific and practical interests and the most powerful means to obtain useful and genetically stable strains for industrial processing. Research has focused on the application of molecular biology method based on the DNA or RNA sequence which permit accurate and fast identify for microbial identification. Polymerase chain reaction (PCR) linked with restriction fragment length polymorphism analyses (PCR-RFLP), is one of molecular biological techniques to identify LAB in several studies (Christensen *et al*., 2004; Claisse *et al*., 2007; Yanagida *et al*., 2008).

Inoculating starter culture into sausage material is used to reduce the ripening time and to ensure the sensory qualities of dry sausages. LAB cause rapid acidification of the raw material through the production of organic acids (mainly lactic acid). LAB also produce ethanol, aroma compounds, bacteriocins, exopolysaccharides, and several enzymes, which are important to enhance shelf life and microbial safety, and pleasant sensory qualities in the end products. The most promising bacteria in starter cultures are those isolated from the indigenous microflora of traditional products due to well adapted to the meat environment and are capable of dominating the microflora of products (Papamanoli *et al*., 2003). LAB, particularly in the genera of *Lactococcus*, *Lactobacillus*, *Pediococcus*, and *Leuconostoc* are involved in the production of fermented food including dairy, vegetable, fruit, meat, and cereal products (Marrug, 1991).

In Thailand, there are many traditional fermented meat products, including mum sausages made from beef, internal organ (liver and spleen), and other ingredients (ground roasted rice, salt, and garlic). The raw material and ingredients are mixed, stuffed into an animal intestine or bladder and then naturally fermented at room temperature for two or three days (Thai Industrial Standards Institute, 2003). However, the fermented meat products, which produce by naturally occurring LAB, often result with inconsistent qualities and unsafe products. Thus, Thai fermented sausage producers are interested in extending the shelf-life of mum product in order to increase the potential market and to satisfy consumer demands.

The developments of Thai fermented meat products have been focus on application of defined starter cultures to improve the products qualities and safety. Therefore, this dissertation is divided into 3 parts: (1) using a modified method to produce mum sausages; (2) isolation and identification of lactic acid bacteria involved in mum sausages and (3) also evaluate their application as starter cultures for mum production.

2. Aim of this study

In summary, most studies involving characterization of LAB isolated from fermented sausages have focused on phenotypic methods. However, various DNA-based methods offer a rapid and more reliable alternative. To select the most suitable starter cultures, which can adapt rapidly as dominate strains during fermentations, it is essential to study the dynamics of the microflora involved. PCR-RFLP is a powerful tool for microbial population studies involving food fermentations, and it has been used to monitor microbial population and dynamics during food fermentations. As discussed by Gänzle *et al*. (1999), bioprotective LAB derived from food may also be useful in the small intestine against food pathogens, as long as they are able to survive the environment of gastrointestinal tract. Moreover, probiotic strains with antimicrobial effects in food act similarly and therefore may be better than commonly used food fermenting bacteria. Thus, the aims of this study were:

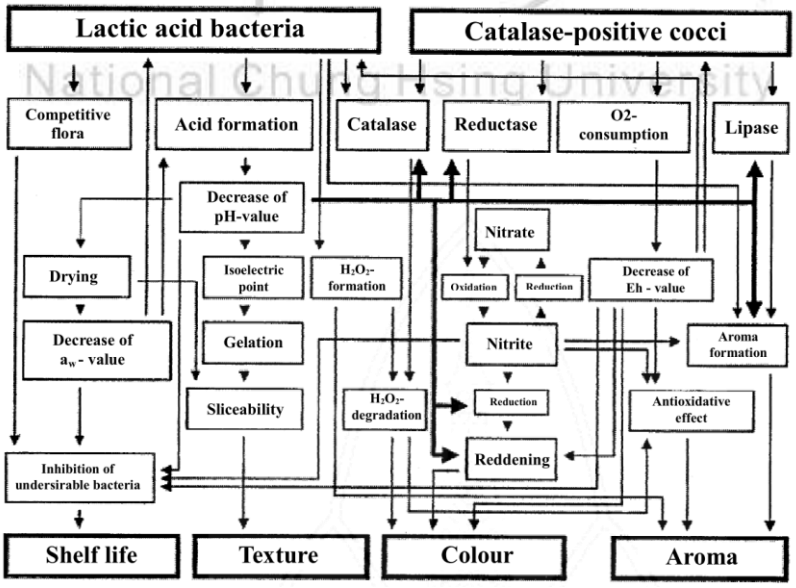
- To compare the physicochemical, microbial, textural, and sensory characteristics of mum sausages between the conventional processing and a modified processing and to evaluate the feasibility of this innovative modify method to improve the qualities and shelf-life (chapter 3).
- To identify the dominant LAB species and to evaluate the variation of LAB strains in the mum sausages manufacturing with the conventional processing or a modified processing and to evaluate the feasibility and efficiency of restriction enzymes, which was used to differentiate lactic acid bacteria in restriction fragment length polymorphism (RFLP) analysis (chapter 4).
- To evaluate the impact of the addition of mixed starter culture (consisting of selected strains of *L. plantarum, L. sakei,* and *Lc. lactis*) and sodium nitrite on the physicochemical, microbial, textural, and sensory properties of mum fermented sausage (chapter 5).

CHAPTER 2

Literature review

1. Fermentation of meat

The preservation of meat by fermentation has been used for thousands of years. Fermentation has been subjected to the action of microorganisms or enzymes so that desirable biochemical changes cause significant modification of the food. However, the term "fermentation" describes a form of anaerobic digestion that generates ATP by the oxidation of certain organic compounds, such as carbohydrates (Adams, 1990). Fermentation causes an increase in organic acids along with a concomitant decrease in pH due to the fermentation of added carbohydrates, i.e., sugar. The primary fermentation product, lactic acid, served to low the pH and contributed to the stability of these sausages against food-borne pathogens and other undesirable microorganisms (Lücke, 2000). Besides lactic acid, there are a variety of products that are produced during fermentation. These include organic acids, carbon dioxide, and alcohols that give distinct flavor and texture of the fermented products (Holzapfel *et al*., 1995). Therefore, fermentation is a biological process and is influenced by many factors that need to be controlled in order to produce a safe and consistent product quality (Fig. 1).



(Buchenhuskes, 1993)

Figure 1. Interactions during the fermentation of sausages caused by the action of lactic acid bacteria and catalase-posotive cocci.

Thailand possesses many kinds of fermented sausages, popular traditional Thai fermented sausages including Nham, E-sarn, and mum sausage. The main ingredients used in Thai fermented sausages are meat, salt, nitrite or nitrate, sugar or cooked rice, and spices. The mixtures are mixed well and stuffed into casing. Natural fermentation is at ambient temperature (about 30℃) for 3 days by predominant bacteria, lactic acid bacteria, until becoming sour (Thai Industrial Standards Institute, 2003).

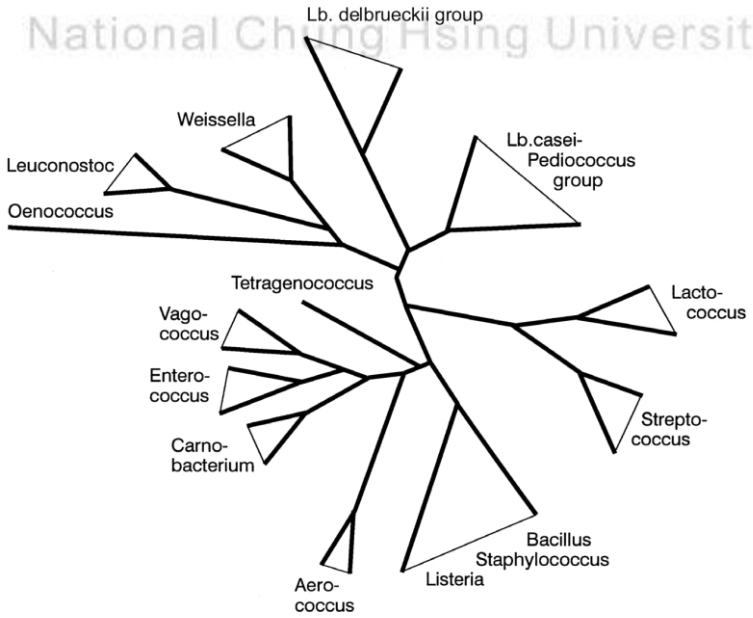
Figure 2. Mum (Thai fermented sausages).

The fermentable carbohydrates are used as carbon sources for the sausage microflora to increase acidity of meat by producing organic acids, mainly lactic acid (Leroy and De Vuyst, 2004). The pH drop caused by organic acids production prevents the growth of spoilage and pathogenic bacteria such as *Salmonella*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Escherichia coli* O157:H7. Sodium chloride added in the raw sausage mixtures affects microorganism growth, interacts and solubilize myofibrillar protein, as well as contributes to the taste of meat products (Yada, 2004). The general consensus of addition of nitrate or nitrite salt to meats is positive contribution to the color, flavor, and shelf life of the product. The curing step has been employed in traditionally fermented sausages to increase the number of lactic acid, color, and flavor forming bacteria (Olesen *et al*., 2004). Nitrite and nitrate salts also inhibit the growth of unwanted microorganisms, particularly *Clostridium botulinum* spores, which can create a lethal toxin. In Thailand, nitrite and nitrate salts should not exceed a maximum level of 125 and 500 ppm (parts per million), respectively (Limsupavanich and Sethakul, 2012). Spices, such as

pepper and garlic, have an impact on flavor, and they may also have antioxidative and antimicrobial effect (Hammes and Knauf, 1994). On the other hand, traditional sausage production depends on natural fermentation; therefore, product quality varies from batch to batch. Unfortunately, if the normal beneficial microflora does not multiply as usual, the product would be spoiled. It may cause illness due to pathogenic microorganisms or their toxins (Woodburn, 1992). To prevent these problems, the starter culture has been widely used in food fermentation.

2. Lactic acid bacteria

Lactic acid bacteria (LAB) ar the most commonly used microorganisms for preservation of food. Their importances are associated mainly with their safe metabolic activity while utilizing available sugar to grow in food (Aguirre and Collins, 1993). LAB consisted of many genus including *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus and Weissella* (Rattanachaikunsopon and Phumkhachorn, 2010; Stiles and Holzapfel, 1997) (Fig. 3).



(Narvhus and Axelsson, 2003)

Figure 3. Schematic, unrooted phylogenetic tree of the lactic acid bacteria, including some aerobic and facultative anaerobic.

The classification of LAB is based on morphology and physiology characteristics such as carbohydrate fermentation profiles, growth at different temperatures, and resistance to salt and high acidity (Rattanachaikunsopon and Phumkhachorn, 2010). However, the classification of most LAB isolates should be possible by using the characterization tests shown in the Table 1. The classification of LAB at the genus level, first divides the LAB, according to morphology, into rods (*Lactobacillus* and *Carnobacterium*) and cocci (all other genera). LAB are mesophilic bacteria, which can grow at temperatures as low as 5°C or as high as 45°C. Additionally, the majority of strains grows at pH 4.0-4.5. Some are active at pH 9.6 and other at 3.2. All LAB produce lactic acid from hexoses. Because LAB lack functional heme linked electron transport chains and a functional Krebs cycle, they obtain energy via substrate level phosphorylation. LAB produce L-lactic acid, D-lactic acid or a mixture of both. However, it should be noted that D-lactic acid is neither metabolized by humans nor recommended for infants and young children due to a typical clinical presentation of D-lactic acidosis, which is encephalopathy and metabolic acidosis (Kang *et al.*, 2006).

Table 1. Differential characteristics of lactic acid bacteria

Character	Rods		Cocci							
	Carno-bacterium	*Lacto-bacillus*	*Aero-coccus*	*Entero-coccus*	*Lactococcus Vagococcus*	*Leuconostoc Oenococcus*	*Pedio-coccus*	*Strepto-coccus*	*Tetragено-coccus*	*Weissella*[b]
Tetrad formation	−	−	+	−	−	−	+	−	+	−
CO_2 from glucose[c]	−[e]	±	−	−	−	+	−	−	−	+
Growth at 10°C	+	±	+	+	+	+	±	−	+	+
Growth at 45°C	−	±	−	+	−	−	±	±	−	−
Growth in 6.5% NaCl	ND[f]	±	+	+	−	±	±	−	+	±
Growth in 18% NaCl	−	−	−	−	−	−	−	−	+	−
Growth at pH 4.4	ND	±	−	+	±	±	+	−	−	±
Growth at pH 9.6	−	−	+	+	−	−	−	−	+	−
Lactic acid[d]	L	D, L, DL[g]	L	L	L	D	L, DL[g]	L	L	D, DL[g]

[a] +, positive; -, negative; +, response varies between species; ND, not determined.

[b] Weissella strains may also be rod-shaped.

[c] Test for homo-or heterofermentation of glucose; negative and positive denotes homofermentative and heterofermentative, respectively.

[d] Configuration of lactic acid produced from glucose.

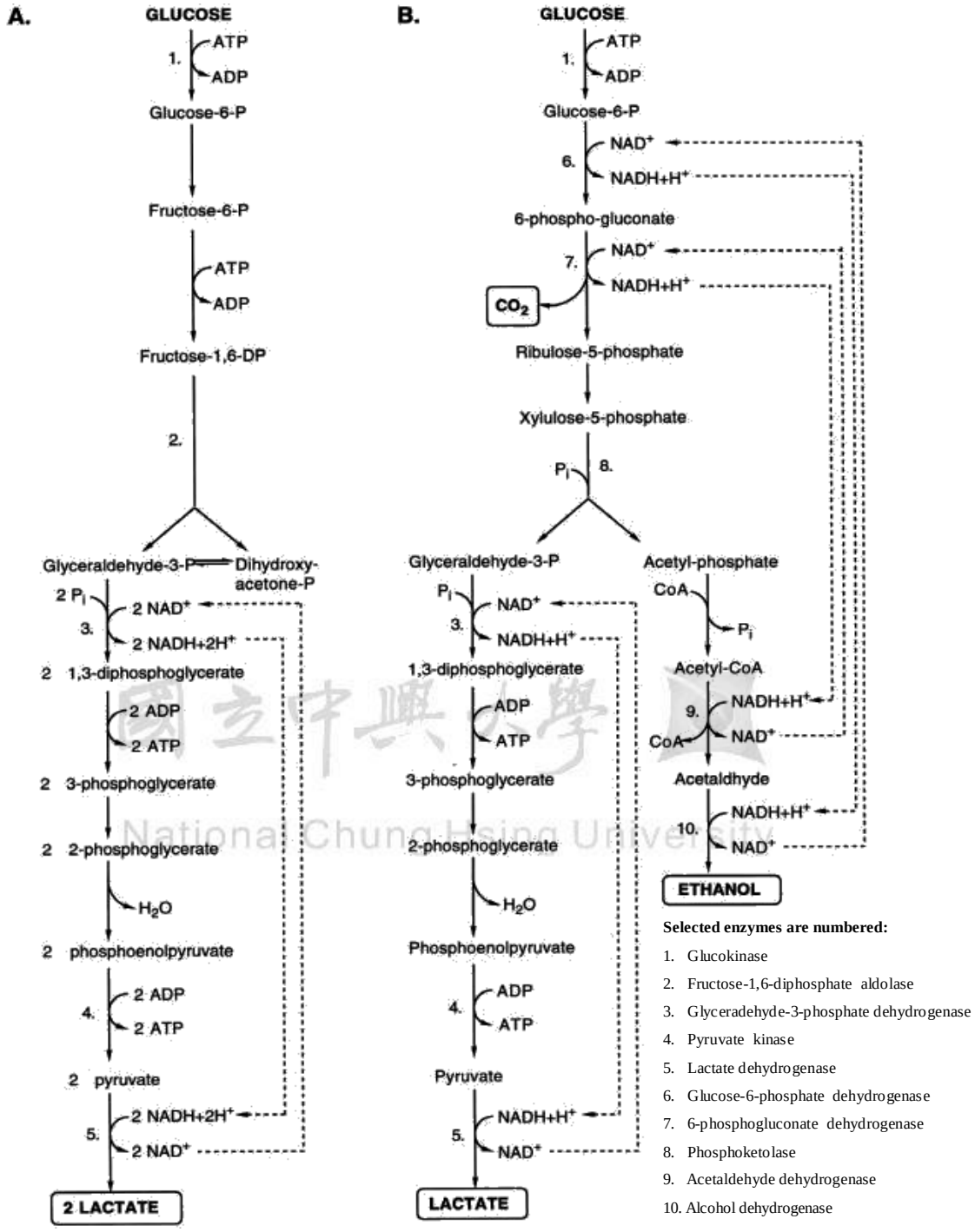
[e] Small amounts of CO_2 can be produced, depending on media.

[f] No growth in 8% NaCl has been reported.

[g] Production of D-, L-, or DL-lactic acid varies between species.

(Axelsson, 1998)

The LAB can be categorized into two groups, homofermentative and heterofermentative, based on the end products formed during the fermentation of glucose (Axelsson, 1998). Homofermentative LAB, such as *Pediococcus, Streptococcus, Lactococcus,* and some *Lactobacillus,* produce lactic acid as the major or sole end-product of glucose fermentation. Homofermentative LAB use the Embden-Meyerhof-Parnas pathway to generate two moles of lactate per mole of glucose and derive approximately twice as much energy per mole of glucose, whereas the heterofermentative LAB (i.e. *Weissella*, *Leuconostoc*, and some *Lactobacilli*) metabolize a glucose molecule via the hexose monophosphate or pentose pathway into lactate, carbon dioxide, and ethanol (Fig. 4) (Aguirre and Collins, 1993; Blandino *et al.*, 2003). These components from LAB could develop a diversity of flavors, aromas, and textures in final fermented foods, and also inhibit undesirable microflora and pathogenic organisms to prevent spoilage and to extend product shelf-life (Rattanachaikunsopon and Phumkhachorn, 2010).



(Axelsson, 1993)

Figure 4. Major fermentation pathways of glucose: (A) homolactic fermentation (glycolysis, Embden-Meyerhof-Parnas pathway); (B) heterolactic fermentation (6-phosphogluconate/ phosphoketolase pathway).

3. Identification of lactic acid bacteria

Due to the important role of LAB in fermented food, LAB have been thoroughly characterized for their metabolic properties, growth performance, resistance to industrial processes, sustain ability in the end product and targeted site of action, shelf-life, etc. Moreover, quality control is crucial and ideal, should be performed on a regular basis to improve technological and safety aspects (Saarela *et al.,* 2000; Temmerman *et al.,* 2004). Therefore, the reliable identification of these microorganisms is great scientific and practical interest (Rodas *et al*., 2003). Over the past decade, a lot of identification techniques are available and displaying differences in discriminatory power, reproducibility, and work load (Temmerman *et al*., 2004; Table 2). Traditionally, LAB have been identified based on phenotypic methods (morphology and biochemical tests). However, phenotypic procedures are labor intensive, time consuming, and inconsistent in results. In response to these limitations, more robust genetic methods based upon molecular biology have been recently developed for the identification and subtyping of bacteria. Molecular techniques are outstanding tools for the typing, taxonomy, and evolution of bacteria involved in food processes (Giraffa and Neviani, 2000). However, genotypic characterizations techniques are also limited by cost, equipment, and databases; therefore, a polyphasic or combined approach is preferred. Table 2 lists a number of frequently applied identification techniques that are discussed in the following paragraphs (Temmerman *et al*., 2004).

Table 2. List of techniques used for the identification of Lactic Acid Bacteria

Technique	Principle	Workload*	Discriminatory power	Reproducibility*	Reference
Phenotypic methods					
Morphological analysis	Microscopic analysis	L	Genus level or less	M	Gonzalez *et al.* (2000)
Physiological analysis	Growth characteristics, simple tests	M	Genus level of less	L	Corsetti *et al.* (2001)
Biochemical characterization	Assimilation and fermentation patterns (API, BIOLOG,. . .)	L	Genus or species level	M	Muyanja *et al.* (2003)
Protein profiling	Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis of cellular proteins	H	Species level	H	Leisner *et al.* (2001)
Genotypic methods					
Specific primers	PCR with group-specific primers	L	Depending on primer	H	Nomura *et al.* (2002)
Sequencing	Determination of gene sequences (16S rDNA. . .)	H	Genus to species level	H	Booysen *et al.* (2002)
RFLP	Restriction Enzyme Analysis (REA) of DNA or PCR amplicons	M	Species to strain level	H	Giraffa *et al.* (2002)
AFLP	Combination of REA and PCR amplification	H	Species to strain level	H	Giraffa and Neviani (2000)
RAPD-PCR	Randomly primed PCR	L	Species to strain level	L	Booysen *et al.* (2002)
Rep-PCR	PCR targeting repetitive interspersed sequences	L	Species to strain level	H	Gevers *et al.* (2001)
PFGE	REA and pulsed-field gel electrophoresis	H	Strain level	H	Ventura and Zink (2002)
Ribotyping	REA and oligonucleotide-probe detection	H	Species to strain level	H	Lyhs *et al.* (2002)
Hybridisation probes	DNA–DNA hybridisation using labeled probes	H	Genus to species level	H	Manero and Blanch (2002)

* L: low; M: Moderate; H: High.

(Temmerman *et al.*, 2004)

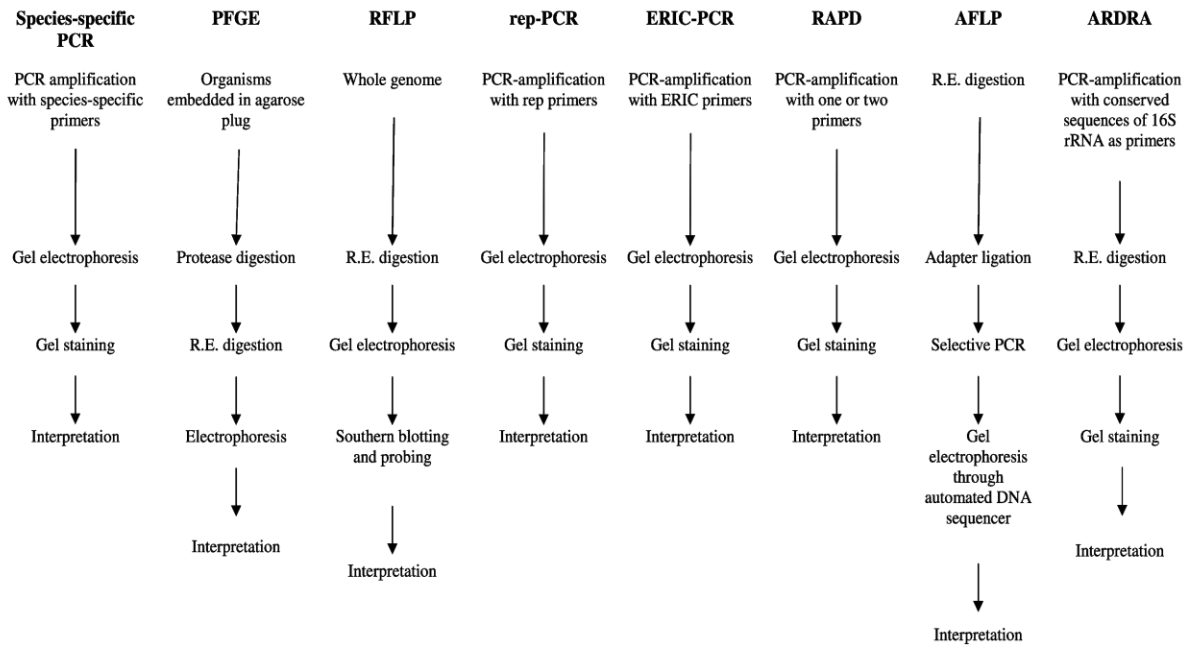
3.1. Phenotypic methods

Phenotyping methods are still being used on a routine basis for the identification of bacteria, due to the fact that they are cheaper compared to genotyping methods and no special skills are required to carry out most tests (O'Sullivan, 1999; Temmerman *et al*., 2004). Phenotyping of bacteria involves all the identification methods that are not based on DNA or RNA (Caplice and Fitzgerald, 1999), which are morphology (e.g. shape, presence or absence of endospores or inclusion bodies), physiology, and biochemical attributes such as conditions required for growth, activities of enzymes, and metabolic activities (Vandamme *et al*., 1996). Although the application of phenotyping method have tried to improve due to laborious and time-consuming (Meroth *et al*., 2003). Additional weakness of phenotyping methods used for LAB strain identification is often ambiguous because LAB have very similar nutritional requirements, grow under similar environment (Rodas *et al*., 2003), strongly adapt to their environment, and share many common attributes (Van Reenen and Dicks, 1996). Other method, based on comparison of the whole cell protein patterns obtained by highly standardized sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE), has been proven to be reliable for comparing and grouping large numbers of closely related isolates (Pot *et al*., 1994). However, SDS-PAGE used for general identification purposes is hampered by the fact that it yields only discriminative information at or below the species level (Vandamme *et al*., 1996). Also, the use of conventional phenotypic methods does not clear identity to species level (Holzapfel *et al*., 1995) and are ineffective in comparing the relatedness between species from different individuals (O'Sullivan, 2000). Therefore, it is essential to use an approach that combines phenotyping and DNA-based techniques with high resolution for identification of LAB associated food fermentation (Vandamme *et al*., 1996).

3.2. Genotypic methods

Identification of LAB isolated from fermented food has been limited by the complexity of the bacterial associations. Additionally, bacterial population involved has similar nutritional and environmental requirements (Schleifer *et al*., 1995). Ideally, these techniques are far more consistent, universally applicable, rapid, reliable, and reproducible and can discriminate even between closely related groups of species, which are otherwise

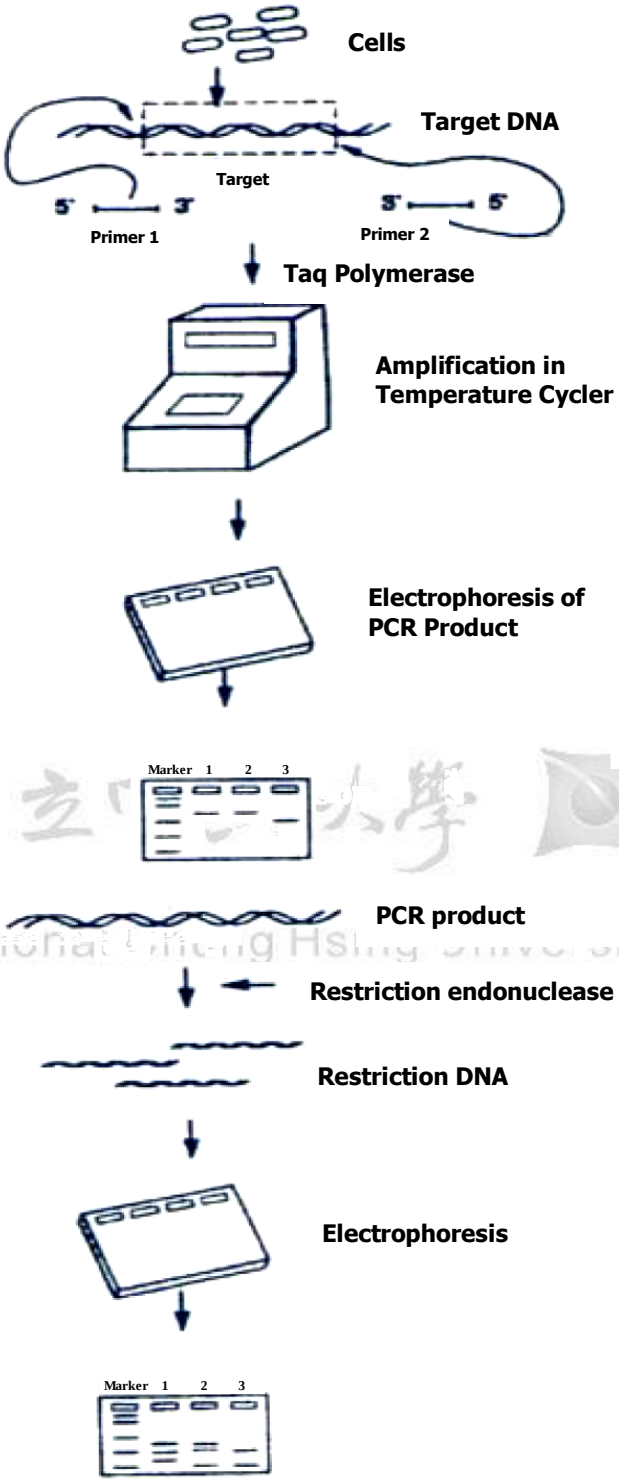
indistinguishable on the basis of their phenotype (Vandamme *et al*., 1996). Many genotypic methods are based on the principle of polymerase chain reaction (PCR), which enables the selective amplification of targeted genes, using primer that correspond to the oligonucleotide sequences present within part of the whole genome of target organisms (Lupski and Weinstock, 1992; Temmerman *et al.*, 2004). Ribosomal RNA or DNA probes specific to particular restriction fragments have been used to develop stable and sensitive typing methods for the *lactobacilli* (Rodtong and Tannock, 1993), especially for 16S rDNA, which has become a very important tool in studying bacterial communities in environmental samples (Ercolini, 2004; Maukonen and Saarela, 2009). The microbial species contain the same length of 16S rDNA gene fragments although their DNA sequences differ (Ercolini, 2004). The 16S rDNA sequence is then compared with a database library by using analysis software. Well-known databases of 16S rRNA gene sequences that can be consulted via the World Wide Web are GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). DNA-based methods that allow identification of LAB to the species and strain level include species-specific PCR (Guarneri *et al*., 2001), restriction fragment length polymorphism (RFLP) (Giraffa *et al*., 2002), pulsed-field gel electrophoresis (PFGE) (Ventura and Zink, 2002), randomly amplified polymorphic DNA-PCR (RAPD-PCR) (Williams *et al*., 1990), repetitive extragenic palindromic-PCR (rep-PCR) (Versalovic *et al*., 1991), amplified ribosomal DNA restriction analysis (ARDRA), amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995), and phylogenetic analyses of housekeeping loci. Comparison of their procedures in the Figure 5 provides a better understanding of their principle.



(Olive and Bean, 1999).

Figure 5. Main steps involved in DNA-based methods for characterization of lactic acid bacteria. R.E. represents restriction enzyme.

Molecular techniques, especially polymerase chain reaction (PCR) linked with restriction fragment length polymorphism analyses (PCR-RFLP), is one of promising molecular biological techniques to differentiate LAB in several studies (Chenoll *et al*., 2003; Christensen *et al*., 2004; Claisse *et al*., 2007; Yanagida *et al*., 2008). Furthermore, PCR-RFLP has been successfully used by Sato *et al*. (2000) to identify 46 LAB isolated from wine and alcoholic drinks. Taylor *et al*. (1997) reported that PCR-RFLP appears to be a reliable method for the routine identification of bacteria and has the potential for providing identifications of bacteria isolates, which are accurate. Additionally, the discriminatory power of these methods is very high (i.e. strain level) making them very useful for typing LAB starter cultures. PCR-RFLP method is widely used for the detection of interspecies variation at the DNA sequence level. It consists in the generation of species-specific band profiles through the digestion of DNA with one or more restriction endonucleases (Temmerman *et al*., 2004). An overview of this approach is shown in Fig. 6.



(Taylor *et al*., 1997)

Figure 6. Schematic overview of the PCR-RFLP method for the microbiological identification of an unknown sample.

4. Role of lactic acid bacteria in food preservation

LAB have long been used in fermentations to preserve the nutritive qualities of various foods. LAB is classified as generally recognized as safe (GRAS) and considered as 'food grade' organisms that are safe to consume (Bredholt *et al*., 2001; Saito, 2004). LAB is applied as starter culture or protective culture in many kinds of fermented foods like yoghurt, cheese, dry sausage, sauerkraut, and sourdough. In meat industry, LAB is widely used as starter cultures for sausage fermentation. They are not only contribute to flavor development, but also inhibit the competing natural flora, which include spoilage bacteria and occasionally pathogens, caused by competition for nutrients and by production of antimicrobial metabolites. The production of one or more antimicrobial active metabolites is part of the complex mechanism. In Table 3, the antimicrobial properties of a number of metabolites from LAB are summarized (Holzapfel *et al*., 1995).

Table 3. Metabolic products of lactic acid bacteria with antimicrobial properties

Product	Main target organisms
Organic acids	
lactic acid	Putrefactive and Gram-negative bacteria, some fungi
acetic acid	Putrefactive bacteria, clostridia, some yeast and fungi
Hydrogen peroxide	Pathogens and spoilage organisms, especially in protein-rich foods
Enzymes	
lactoperoxidase system with H_2O_2	Pathogens and spoilage bacteria (milk and dairy products)
lysozyme (by recomb. DNA-technology)	Undesired Gram-positive bacteria
Low-molecular metabolites	
reuterin (3-OH-propionaldehyde)	Wide spectrum of bacteria, moulds and yeast
diacetyl	Gram-negative bacteria
fatty acids	Different bacteria
Bacteriocins	
nisin	Some LAB and Gram-positive bacteria, notably endospore-formers
other	Gram-positive bacteria, inhibitory spectrum according to producer strain and bacteriocin type

(Holzapfel *et al*., 1995)

4.1. Organic acid

During fermentation, production of lactic acid contributes to a major safety factor in fermented food. Holzapfel *et al*. (1995) reported that the characteristic of organic acids (such as lactic acid and acetic acid) produced by LAB may reduce pH to a level where putrefactive (e.g. *Clostridia* and *Pseudomonads*), pathogenic (e.g. *Salmonellae* and *Listeria sp*.), and toxinogenic bacteria (e.g. *Staphylococcus aureus*, *Bacillus cereus*, and *Clostridium botulinum*) will be either inhibited or destroyed. The antimicrobial action of these acids is related to the ability of the undissociated acid molecules to penetrate through the bacterial plasma membrane. In the cytoplasm, the acid dissociates to release protons and conjugate bases with higher pH. This disrupts the membrane proton-motive force, thus disabling the energy-yielding and transport process dependent upon it (Bearson *et al*., 1997; Savard *et al*., 2002). Blom and Mortvedt (1991) indicated that acetic acid is the strongest inhibitor and has a wide range of inhibitory activity.

4.2. Hydrogen peroxide

In the presence of oxygen, LAB are able to generate hydrogen peroxide (H_2O_2) through the action of flavoprotein-containing oxidases, NADH oxidases, and superoxide dismutase. Accumulation of hydrogen peroxide can occur and inhibit some microorganisms. Inhibition is mediated through the strong oxidizing effect on membrane lipids and cell proteins (Mayra-Makinen and Bigret, 2004). Brashears *et al*. (1998) showed that H_2O_2 from *Lactococcus lactis* is the main factor responsible for the antagonistic action toward *E. coli* 0157: H7 on refrigerated raw chicken meat.

4.3. Carbon dioxide (CO_2)

Carbon dioxide is mainly produced by heterofermentative LAB from hexose. It contributes to a reduced Eh and is directly toxic to a number of aerobic putrefactive bacteria but may promote the growth of others (Holzapfel *et al.*, 1995). Moreover, the effect of CO_2 can cause the growth inhibition of many food spoilage microorganisms, especially Gram-negative psychrotrophic bacteria (Farber, 1991).

4.4. Diacetyl

A number of primary metabolites of low molecular weight are known for their relatively potent antimicrobial activities. Diacetyl (2, 3-butanedione) is known to be

responsible for the characteristic aroma and flavor of butter. It is produced by some *Lactococcus, Leuconostoc,* and *Pediococcus spp.,* etc. during degradation of citric acid (Holzapfel *et al*., 1995). Although diacetyl is identified as a flavor compound, but its antibacterial action is noted by many researchers. Its mode of action is believed to interference with the utilization of arginine (Caplice and Fitzgerald, 1999). Kang and Fung (1999) suggested that diacetyl could be used as a food ingredient during meat fermentation to control *E. coli* 0157:H7 and *Salmonella typhimurium* without harmful effects on the growth and acid production of starter culture.

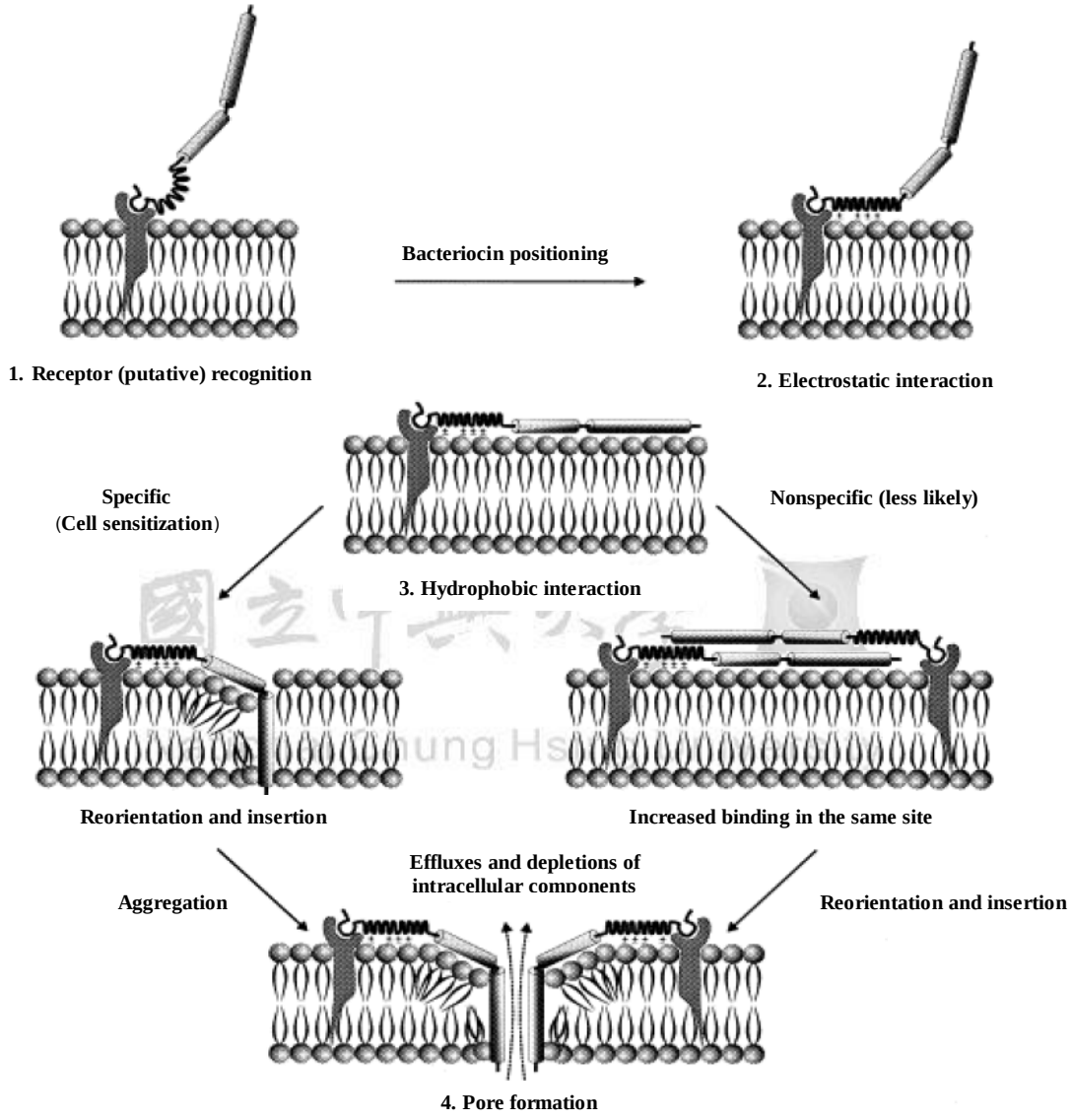
4.5. Reuterin

Reuterin is a low molecular weight antimicrobial substance produced by *Lactobacillus reuteri*, when it is grown anaerobically on a mixture of glucose and glycerol or glyceraldehyde, dependent on coenzyme B_{12} (Ouwehand, 1998). Reuterin has a broad spectrum of antimicrobial activity against certain bacteria, yeast, fungi, and protozoa (Rattanachaikunsopon and Phumkhachorn, 2010).

4.6. Bacteriocins

Bacteriocins from LAB are ribosomally produced peptides (usually 30–60 amino acids) that display potent antimicrobial activity against certain Gram positive organisms (O'Sullivan *et al*., 2002), and are produced by many different bacteria species, including many member of the LAB. *Lactobacilli* that being isolated from sausage frequently produce bacteriocins or bacteriocin-like compounds, as has been shown from *L. sakei*, *L. curvatus*, *L. plantarum*, *L. brevis*, and *Lb. casei* (Leroy *et al*., 2006). Some bacteriocins produced by LAB inhibit not only closely related species but also food-borne pathogens such as *Listeria monocytogenes*, *Clostridium botulinum*, and *Staphylococcus aureus* (Lewus *et al.*, 1991). It also inhibit other Gram-positive spoilage microorganisms including *Bacillus sp*. and *Enterococcus faecalis* (Delves-Broughton, 1990). *Lc. lactis* strain WNC 20 is able to produce the bactericocin nisin-Z originally isolated from Nham (Noonpakdee *et al*., 2003). Bacteriocin-producing *Lc. lactis* strains showed a potential use as protective culture to improve the food safety of the fermented product (Leroy *et al*., 2006). They functioned by absorption to the membrane of receptors on the target cells, insertion, and pore formation (Ouwehand, 1998) (Fig. 7). Almost all bacteriocins have a net positive charge at

neutral or slightly acidic pH and usually contain stretches of hydrophobic and/or amphiphilic sequence (Eijsink *et al*., 2002).



(Ennahar *et al*., 2000).

Figure 7. Schematic representations for bacteriocin insertion and formation of hydrophilic pores.

Bacteriocins of LAB are classified into four groups as shown in Table 4: I) the lantibiotics, low molecular; II) small hydrophobic heat-stable peptides; III) large heat-labile proteins; and IV) complex bacteriocins showing the complex molecule of protein with lipid and/or carbohydrate (Ouwehand, 1998). Bacteriocins, produced by LAB, may be considered as natural preservatives or biopreservatives. Biopreservation refers to the use of antagonistic microorganisms or their metabolic products to inhibit or destroy undesired microorganisms in food to enhance food safety and extend shelf life (Schillinger *et al.*, 1996). Three approaches are commonly used in the application of bacteriocins for biopreservation of food: (1) inoculation of food with LAB that produced bacteriocin in the products; (2) addition of purified or semi-purified bacteriocins as food preservatives; and (3) use of a product previously fermented with a bacteriocin-producing strain as an ingredient in food processing (Schillinger *et al.*, 1996; Chen and Hoover, 2003).

Table 4. Classification of bacteriocins from lactic acid bacteria

Type	Structure	Heat stability	Antibacterial spectrum	Example
Lantibiotics	small (< 5 kDa) unusual amino acids (e.g. lanthionine)	+	medium to broad	nisin lacticin 481
Peptide bacteriocins	small (< 10 kDa) no lanthionine	+	medium to broad	pediocin AcH sakacin A leucocin UAL 187
Protein bacteriocins	large (> 10 kDa) no lanthionine	-	narrow	helveticin J caseicin 80
Complex bacteriocins	Glyco- and/or lipid moiety	+	medium	leuconocin S pediocin SJ-1

(Holzapfel *et al.*, 1995)

5. Lactic acid bacteria as functional starter cultures

A meat starter culture can be described as viable microorganisms added directly to meat in order to improve the storage quality, safety and/or enhance consumer acceptability of meat product (Kunawasen, 2000). A wide variety of microorganisms, mainly LAB, has been selected for using in meat ecosystems to improve the quality and safety of the final

product (Baruzzi *et al*., 2006). Meat starter cultures are selected bacteria that have been isolated, purified, and grown in large numbers under controlled conditions (Bonomo *et al*., 2008). Starter cultures can be classified into the following groups: (1) lactic acid producing cultures; (2) color fixing and flavor forming cultures; (3) surface coverage cultures; and (4) bio-protective cultures or bacteriocin-producing starter cultures (Leroy *et al*., 2006). The advantages of starter cultures are complete fermentation, control fermentation rate, reduce fermentation time and off-flavors, form more consistent flavor characteristics, and improve quality and profitability. Starter cultures also provide safety by competing and inhibiting the growth of undesirable bacteria (Hugas and Monfort, 1997).

Microorganisms belonging to the *lactobacillus* group, i.e. *L. plantarum*, *L. sakei*, *L. curvatus* and *L. pentosus*, have been employed in meat fermentation (Ammor *et al*., 2005; Visessanguan *et al*., 2006a). The performance of *L. plantarum* and *L. pentosus* as meat starter cultures for manufacture of the Scandinavian-type fermented sausage was evaluated. The sausages inoculated with *L. plantarum* and *L. pentosus* rapidly reached final pH of 4.8-5.0, which inhibit pathogens and spoilage bacteria, and facilitate production of an acidic taste (Klingberg *et al*., 2005). In the study of Lee *et al*. (2006), *L. plantarum* isolated from kimchi had an ability to adapt to the complex environment of fermented sausage, which allowed them to act as starter cultures and natural preservatives in sausage production.

Basic flavor results from the interaction of taste (mainly determined by lactic acid production and the pattern of peptides and free amino acids resulting from tissue-generated proteolysis) and aroma (mainly determined by volatile components derived from bacterial metabolism and lipid autoxidation) (Leroy *et al*., 2006). Lactic and acetic acids are often suggested to be the major contributors to the acid aroma and taste and the development of texture. The LAB neither possessed high proteolytic nor lipolytic capabilities (Visessanguan *et al*., 2006b). Various LAB are often used as starter cultures in production of dry fermented sausages due to their nitrate reductase activity and flavor enhancing capacity. In a previous study (Møller *et al*., 2003), *L. plantarum*, *L. fermentum*, and *P. acidilactici* had ability to generate NO that could form the basis for production of cured meat products without the use of nitrate/nitrite. Kawahara *et al*. (2006) found that acceptable shelf stability and color in cured meat products might be attained by the use of

L. sakei as a starter culture, instead of nitrite. Therefore, NO producing bacterial strains could become an alternative to the usage of nitrate and nitrite in fermented meat product. Additionally, *Staphylococcus* often possesses catalase activity, which could indirectly alter flavor formation by preventing chemical oxidation of fatty acids (Talon *et al*., 1999, Tjener *et al*., 2003). *Staphylococci*, in particular *Staphylococcus xylosus* and *S. carnosus*, modulate the aroma through the conversion of amino acids (particularly the branched-chain amino acids leucine, isoleucine, and valine) and free fatty acids. In addition, additives such as nitrate, nitrite, or ascorbate, pre-cultivation parameters and environmental factors clearly influence the generation of aroma compounds (Leroy *et al*., 2006).

Table 5. Typical examples of functional starter cultures or co-cultures and their advantages for the food industry

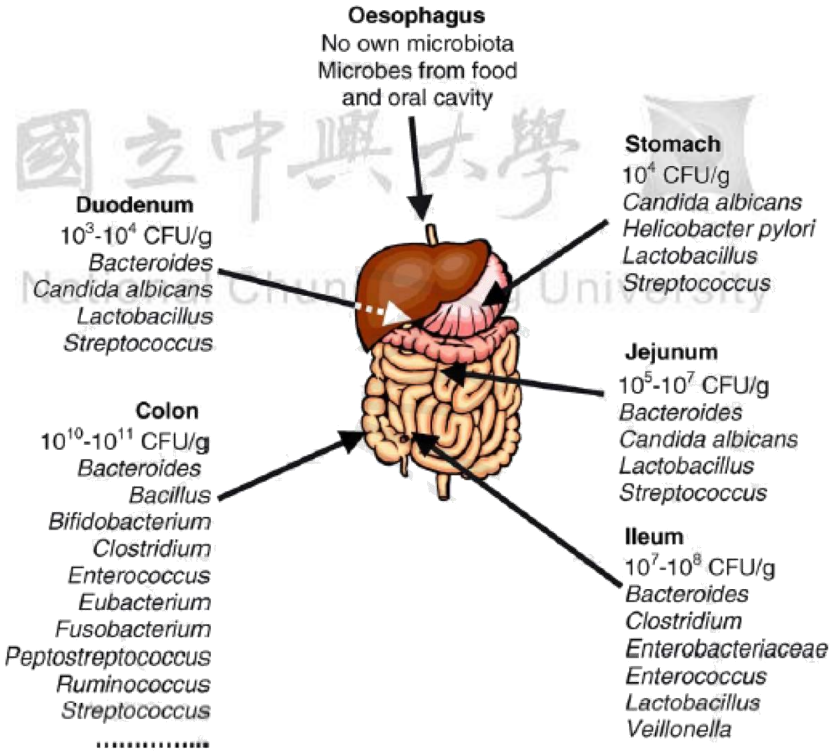
Advantage	Functionality	Lactic acid bacteria[a]
Food preservation	Bacteriocin production	
	- Dairy products	*L. lactis subsp. lactis, Enterococcus spp.*
	- Fermented meats	*Lb. curvatus, Lb. sakei, P. acidilactici, E. faecium*
	- Fermented olives	*L. plantarum*
	- Fermented vegetables	*L. lactis*
Organoleptic	Production of exopolysaccharides	Several *lactobacilli* and *streptococci*
	Production of amylase	Several *lactobacilli*
	Aroma generation	Several strains
	Enhanced sweetness	
	- Homoalanine-fermenting starters	*L. lactis*
	- Galactose-positive/glucose-negative starters	*Lb. delbrueckii subsp. bulgaricus, S. thermophilus*
	- malolactic fermentation	*O. oeni*
Nutritional	Production of nutraceuticals	
	- Low-calories sugars	*Lb. plantarum*
	- Production of oligosaccharides	*L. lactis*
	- Production of B-group vitamins	*L. lactis, S. thermophilus*
	Reduction of toxic and anti-nutritional compound	
	- Production of L(+)-lactic acid isomer	L(+)-lactic acid-producing strains
	- Removal of lactose and galactose	*S. thermophilus*
	- Removal of raffinose in soy	Several strains
Technological	Bacteriophage resistance	Several strains
	Prevention of overacidification in yoghurt	Lactose-negative *Lb. delbrueckii subsp. bulgaricus*
	Autolysing starters	
	- Phage-mediated	*L. lactis subsp. lactis*
	- Bacteriocin-induced	*L. lactis*

[a] *E.=Enterococcus, L.=Lactococcus, Lb.=Lactobacillus, O.=Oenococcus, P.=Pediococcus, S.=Streptococcus*

(Rattanachaikunsopon and Phumkhachorn, 2010)

6. Lactic acid bacteria as probiotics

LAB have been received major attention for probiotic activity and generally considered as good probiotic organisms (Klein *et al*., 1998; Saavedra, 2001; Sullivan *et al*., 1992). *Lactobacillus spp.* and *Bifidobacterium spp.*, prominent members of the commensally intestinal flora, are commonly studied as probiotic bacteria. They are able to reduce lactose intolerance, prevent diarrhea, decrease blood cholesterol, increase immune responses and prevent cancer (Marteau and Rambaud, 1993; Soomro *et al*., 2002). For probiotic microorganisms to exert beneficial health, it is important that the culture, which is added at the time of manufacture, remains viable at high concentrations during the relevant shelf-life or storage period.



(Isolauri *et al*., 2004)

Figure 8. The numerically dominant microbial genera in the adult human gastrointestinal tract.

Microorganisms used as probiotics are *Lb. acidophilus, Lb. plantarum, Lb. casei, Lb. casei subsp. Rhamnosus, Lb. delbreuckii subsp. bulgaricus, Lb. fermentum, Lb. reuteri, Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. cremoris, Bifidobacterium bifidum, Streptococcus salivarius subsp. thermophilus, Enterococcus faecalis*, and *E. faecium* (Soomro *et al*., 2002). In general, the minimum concentration of probiotic microorganisms necessary to exert a beneficial effect remains unclear. However, the therapeutic minimum dose of 10^5 viable cell/g or ml of product has been proposed (Lee and Salminen, 1995).

Most scientists agree that probiotic strains should survive transit in the GI tract well and exert beneficial effects in the lower small intestine and the colon. Although there are convincing data on beneficial immunological effects from dead cells (Mottet and Michetti, 2005). LAB isolated from traditional fermented dairy products in Inner Mongolia, China, were supplied to bile tolerance trials as indicator tests of probiotics. Results showed that *L. plantarum* strain 301102 exhibited the growth rate of 60% after 6 h in MRS broth containing 0.3% oxgall and the survival rate of 71% after 3 h in artificial gastric juice adjusted pH 2.0, and could grow in artificial intestinal juice. This suggested that the strain might be grown in intestine after overcome low pH in stomach and bile acid. As a characteristic of this strain, the tolerance to the bile was extremely high by growing in MRS broth containing 20% oxgall (Tsuda *et al*., 2008). Although, the primary role of food is to provide enough nutrients to fulfill human metabolic requirements, and beyond nutrition in the conventional sense, food may modulate various functions in the body. Functional foods containing probiotic are subset (Stanton *et al*., 2001).

CHAPTER 3

An innovative method for the mum (Thai fermented sausages) preparation with acceptable technological quality and extended shelf-life

1. Abstract

Freshly-manufactured mum sausages were produced by two processing methods (process I: fermented and stored at ~30°C for 14 days; process II: fermented at ~30°C for three days, vacuum-packaged and stored at 4°C until day 28). Physiochemical, microbial, textural, and sensory properties of the sausages were analyzed. The results showed that dehydration was more extensive in process I samples, and resulted in lower moisture content and water activity than process II samples. Significant decreases in pH values and increases in lactic acid were observed in both samples at day 3. The total microflora and lactic acid bacteria counts increased rapidly during the fermentation and then decreased as *Enterobacteriaceae* counts decreased. Extensive dehydration resulted in tough textures and unacceptable sensory qualities for the process I samples. In conclusion, three days of fermentation, with vacuum-packaging, ripening and storage at 4°C, were possibly to produce mum sausages with better qualities and more extended shelf life.

Keywords: microbial properties, physiochemical properties, sensory characteristics, Thai fermented sausage (mum), vacuum-package

2. Introduction

Mum is a typical dry fermented sausage abundantly produced and commonly consumed in the north-eastern region of Thailand. The basic ingredients of mum are beef, liver, spleen, ground roasted rice, salt, and garlic. The mixture is stuffed into an animal intestine or bladder and then held at room temperature for two or three days (Thai Industrial Standards Institute, 2003). According to González-Fernández *et al*. (2006), the manufacturing of fermented sausages can be divided into three stages. First, in the preparation period, the raw materials and ingredients are minced, blended, and stuffed into casings. In the fermentation period, lactic acid bacteria, which produce lactic acid and decrease pH values in the products, contribute to further curing color formation, muscle protein coagulation, and increasing firmness and cohesiveness of products. Finally, in the subsequent ripening period, the product is further dried and develops the final characteristics of fermented flavor and texture.

In Thailand and many other areas, many traditional fermented meat products, including mum sausages, were conventionally naturally-fermented (which means without addition of starter cultures during manufacturing). When manufacturing these traditional fermented products, most of them were seasoned and processed according to the traditional know-how. The production of these traditional products commonly replies on the microflora, which naturally colonizes the processing unit's environment (Talon *et al*., 2007).

However, the productions of fermented meat products which use naturally occurring lactic acid bacteria often result in products with inconsistent qualities and even unsafe products, if they are improperly handled. Furthermore, such microflora might contain some useful microorganisms for the fermentation flavor and texture development of the products, but may also include some spoilage species or sometimes even some kinds of pathogenic microorganisms (Talon *et al*., 2007). Wiriyacharee *et al*. (2003) indicated that mum produced by using starter cultures had better qualities than traditional products in terms of sourness and lactic acid production. Furthermore, many efforts, such as good hygiene practices, packaging, and addition of antioxidants and preservatives have been applied to improve the products qualities and safety (Rubio *et al*., 2008). Additionally, in order to have the final products with more stable and consistent qualities, many products, especially those produced in large industrial-production systems nowadays, are often manufactured under controlled conditions (i.e. controlled temperature and humidity, application of starter cultures, and etc).

Sensory qualities, shelf-life, and safety of fermented products are the result of an extremely complex interaction between physical, chemical, microbiological, and textural changes during fermentation and ripening stages (Demeyer and Toldra, 2004). In recent years, the principles of hazard analysis and critical control point (HACCP) models have been developed for the production of Thai fermented sausages (Paukatong and Kunawasen, 2001). Nowadays, food producers have been urged to develop products with desirable qualities and extended shelf-life in order to satisfy consumers' demands and possibly to increase a potential market.

Therefore, the objectives of this study were to compare the physicochemical, microbial, textural, and sensory characteristics of mum sausages which were manufactured by the conventional method (i.e. fermentation, ripening, and storage at ~30°C) or a modified method (i.e. fermentation at ~30°C for three days, vacuum-packaged, ripening, and storage at 4°C), and to evaluate the feasibility of this innovative method to improve the qualities and extend the shelf life of mum sausages.

3. Materials and methods

3.1. Sausage preparation

Freshly-manufactured mum sausages, which were made according to the traditional techniques by mixing minced beef (60% w/w), minced bovine liver (15% w/w), minced spleen (15% w/w), roasted rice powder (4.2% w/w), garlic (4.2% w/w), salt (1.6% w/w), and some spices and seasonings (trace amounts), and then extruded through a stuffing horn into intestine casings with a diameter of 3.0 cm and a length of 15 cm (approximately 250-300 g each), were collected from local meat factories in Chaiyaphum (CP) and Khon Kaen (KK) provinces in Thailand, transported to the lab, and assigned into the two follow-up processing methods. The main ingredients of sausages produced in these two areas were most likely the same, except for some minor difference in spices and seasonings they used. For process I which represented the conventional method, after collection, sausages were hung vertically on stainless steel hangers at the environment with approximately 30 ± 2°C and 65 ± 2% relative humidity until day 14 without any packaging. For process II which represented the modified method, after collection, sausages were hung vertically on stainless steel hangers at the environment with approximately 30 ± 2°C and 65 ± 2% relative humidity until day 3 for the fermentation purpose, then vacuum-packaged in a vacuum bag (laminate of Nylon/LLDPE, Chun I Gravure Co. Ltd., Taichung, Taiwan), and stored at 4°C until day 28. Sausages were sampled on day 0 (after stuffing), day 3 (end-fermentation), day 14 (end-ripening process), and day 28 (post-ripening storage) for analyses (González-Fernández *et al*., 2006).

3.2. Physicochemical analysis and instrumental color evaluation

Proximate compositions of samples, including moisture, crude protein, crude fat,

and ash contents, were measured according to the AOAC (1990) methods. Crude protein was measured by the Kjeldahl method, using a digester (Model 2006, Foss tecator, Sweden) and a distillation unit (Model 2100, Foss tecator, Sweden). Crude fat was measured using a fat extractor (Sotec System HT 1043 Extraction Unit, Tecator Co., Sweden) with ethyl ether as a solvent and extracted for 16 h. Water activity (a_w) was measured using a water activity analyzer (Aqualab, series 3, Decagon Devices Inc., USA). Ten gramme samples were blended with 90 ml distilled water in a polyethylene bag for 2 min using a laboratory blender, and then the pH value of mixture was measured using a pH meter (Weilheim, Germany). Titratable acidity was determined as percentage of lactic acid by titrating with 0.1 N NaOH, using phenolphthalein as an indicator (AOAC, 1990). Color parameters of samples were measured with a Konica Minolta Chroma Meter (an 8-mm port size, D65 illuminant, and 10-degree observer, CR-400, Osaka, Japan). The instrument was first standardized with a white calibration plate. Six measurements of surface reflectance which expressed as L* (lightness), a* (redness), and b* (yellowness) values were performed on the slices of sausages with a thickness of approximately 3 cm and averaged. Additionally, a total color difference (△E*) and chroma difference (△C*) were calculated as $\Delta E^* = [(L^*_1 - L^*_2)^2 + (a^*_1 - a^*_2)^2 + (b^*_1 - b^*_2)^2]^{1/2}$ (Nanke *et al*., 1998) and $\Delta C^* = [(a^*_1 - a^*_2)^2 + (b^*_1 - b^*_2)^2]^{1/2}$ (Choi *et al*., 2011), respectively.

3.3. Microbiological analysis

Microbial qualities were determined accordingly (APHA, 2001). At a specified sampling time, 25-gram samples were aseptically placed in a sterile bag, which contained 225 ml of 0.85% NaCl solution, and homogenized with a stomacher (Stomacher, Oskon Co., Ltd., Thailand) for 2 min. Serial dilutions were then made. Plate count agar (Merck, Dram Stadt, Germany) and violet red bile agar (Merck, Dram Stadt, Germany) were used for enumeration of total plate counts and *Enterobacteriaceae* counts, respectively, using the pour plate method. Total microflora and *Enterobacteriaceae* were incubated at 35°C for 24 h. For lactic bacteria counts, dilutions were poured into MRS agar (Merck, Dram Stadt, Germany), placed in an anaerobic jar (BBL GasPak System, USA) and incubated at 37°C for 48 h. The microbial counts were expressed as $\log_{10}$ colony forming units (CFU) per gram of sample.

3.4. Textural and sensory evaluation

In texture profile analysis (TPA), a texture analyzer (Stable Micro Systems TA-XT2i, Surrey, UK) equipped with a cylindrical probe P/25 was applied to determine texture parameters including hardness, springiness, adhesiveness, cohesiveness, and chewiness of samples. This procedure involved cutting five slices of sausage samples with approximately 1.5 cm in height and 2.0 cm in diameter, after discarding the external layer. Samples were allowed to equilibrate to room temperature and then compressed twice to 50% of the original height. Force–time deformation curves were obtained with a 25 kg load cell which applied at a cross-head speed of 1 mm/s (Bourne, 1978). At the end of fermentation and storage stages (i.e. day 3 and day 28, respectively), samples were served to a sensory panel, which consisted of 15 panelists. Sensory attributes, including color, flavor, texture, sourness, saltiness, and overall acceptability, were evaluated using a 1 to 7-point scale test, with 1 and 7 representing dislike very much and like very much, respectively.

3.5. Statistical analysis

In this study, it was sampled for 3 times during 03/2010-05/2010. The result of analyses which depend on processing methods and processing time were analyzed according to a completely randomized design with three replicates (3, 3, 5, and 5 samples for microbial, chemical, color, and texture analysis, respectively). All data were subjected to variance analyses and differences between means of processing time were evaluated by Duncan's multiple range test and means of processing methods were evaluated by t-test using Statistical Analysis System's Procedures (Version 9.1, SAS Institute Inc., Cary, NC) with a 5% level of significance.

4. Results and discussion

4.1. Physicochemical analysis

In this study, sausages were sampled on day 0 (after stuffing), day 3 (end-fermentation), day 14 (end-ripening), and day 28 (post-ripening storage) for analyses (González-Fernández *et al*., 2006). However, due to too tough texture which made process I samples not suitable for consumption after storage at ambient temperature for 14 days, only process II samples were evaluated on day 28. Changes in proximate composition and water

activity of mum sausages are presented in Table 6. Moisture contents of all samples decreased significantly from 70-72% on day 0 (after stuffing) to 52-55% on day 3 (end-fermentation). Moisture contents of process I samples continuously decreased to approximately 39% on day 14 (end-ripening), while the moisture contents of process II samples slightly decreased to 51-53% on days 14 and 28. Less moisture evaporation which was due to the protection of vacuum-packaging resulted in less intensive dehydration of the product and higher moisture contents in process II samples. This higher moisture content was also coincided with the higher water activity in process II samples. Crude protein and crude fat contents of the samples increased with storage time for both processes due to the dehydration of moisture.

The results in table 6 showed that the averaged water activity (a_w) of process I samples decreased significantly from 0.98 on day 0 to 0.96 on day 3, significantly and even more dramatically decreased to approximately 0.86 on day 14. According to Roig-Sagués *et al.* (1999), a_w in Spanish ripened sausages hardly changed during the first nine days of elaboration and the authors explained that this slight change in a_w (0.97 to 0.95) was probably due to the loss of free water which hardly influenced the decrease in a_w. Afterwards, the a_w was reduced markedly to 0.85 due to the loss of bound water. Continuous decreases in a_w but with a comparatively less degree, was also observed in process II samples in this study. This resulted in process II samples to have significantly higher a_w than that of process I samples on day 14. Due to the intensive moisture dehydration in process I samples, it resulted in a tough texture which was not suitable for sensory evaluation and consumption. Only the a_w of process II samples was determined on day 28 (post-ripening storage) and it was 0.93. Less reduction during storage and significantly higher a_w in process II samples when compared to that of process I samples was because less moisture dehydration occurred due to the less moisture permeability in the vacuum-packaging materials and it agreed with many other studies (Rubio *et al.*, 2007; Rubio *et al.*, 2008). In this study, steadily decreases in *Enterobacteriaceae* counts and a_w were observed during processing and it agreed with Roig-Sagués *et al.* (1999) who reported a significant correlation (R^2=0.98) was observed between the a_w and the development of enterobacteria in a Spanish ripened sausage.

In this study, the pH values of samples decreased significantly ($P < 0.05$) on day 3 (end-fermentation) for the samples of both processes; after day 14, process I samples had a significantly higher pH value than that of process II samples (Table 7). A pH change pattern which consisted of a rapid decrease at first, followed by a steady or slow decrease, and then finally a rise during the processing and storage time was observed in a Spanish dry-cured sausage (González-Fernández *et al*., 2006**)**. Many researchers have reported that this final pH increase in meat and meat products could be due to an accumulation of non-protein nitrogen and amino acid catabolism products (Pérez-Alvarez *et al*., 1999), the growth of spoilage bacteria (Ahmad and Srivastava, 2007) or the formation of some alkaline compounds, such as biogenic amines (Roig-Sagués *et al*., 1999).

In order to ensure the shelf-life and safety of fermented meat products, Demeyer and Toldra (2004) indicated that a fast drop to an acidic pH (< 5.0 after 3 days) at temperatures higher than 20°C should be achieved. In this study, KK-I had pH of 5.1 after fermentation whereas the pH values of process II samples during refrigerated storage for up to 28 days were low enough (i.e. < 5.0) to avoid the growth of pathogenic microorganisms and stabilize the final product (Cabeza *et al*., 2009). As expected, the values of titratable acidity had an inverse relationship to pH values during the process. Therefore, with a rapid decrease in pH values during fermentation, titratable acidity increased significantly ($P < 0.05$) on day 3 and then remained stable.

4.2. Instrumental color evaluation

In this study, L* (lightness) and b* values (yellowness) of process I and II samples increased during processing except for some minor exceptions (Table 8). In addition to acidification of meat proteins which increasing the light scattering, Visessanguan *et al*., (2005) explained that shrinkage of the myofilament lattice also increases the light reflected from the meat, and thus meat becomes more opaque and paler. The a* values (redness) of samples decreased during processing, especially ($P < 0.05$) during fermentation. Gøtterup *et al*., (2008) indicated that nitrosylmyoglobin was the major pigment responsible for the characteristic bright red cured color in fermented sausages. Some authors have reported that the decrease in a* values during the ripening of fermented sausages was probably due to the effect of lactic acid partially or totally denatured the different states of myoglobin, such as

myoglobin, oxymyoglobin, and nitrosomyoglobin (García-Marcos *et al*., 1996).

In this study, it was found that process II samples had lower total color difference and chroma difference than that of process I samples (△E*: CP-I = 1.92 vs. CP-II = 1.40 and KK-I = 3.02 vs. KK-II = 1.27, (△C*: CP-I = 1.26 vs. CP-II = 1.22 and KK-I = 1.91 vs. KK-II = 0.76) between day 3 (end-fermentation) and day 14 (end-ripening). This result has demonstrated that mum sausages that were produced by the modified method (i.e. vacuum-packaged and refrigerated storage) had less color change when compared to that of sausages produced by the conventional method. This less discoloration was possibly due to less oxidation occurring during processing. Gøtterup *et al*. (2008) stated that the oxidative discoloration of fermented sausage was probably due to the conversion of nitrosylmyoglobin to the brown metmyoglobin, and explained this quality deteriorating is related to the oxygen partial pressure and myoglobin reducing systems and is most likely related to the subsequent lipid oxidation of products.

4.3. Microbial qualities

Microbial changes of samples during storage are presented in Fig. 9. The total plate counts (TPC) of samples increased significantly from 6.2-7.8 log CFU/g on day 0 (after stuffing) up to 8.5-9.7 log CFU/g on day 3 (end-fermentation), and then decreased significantly to 5.8-8.2 log CFU/g on day 14 (end-ripening). Further reduction in TPC was observed in process II samples on day 28 (post-ripening storage). Similarly, the lactic acid bacteria (LAB) counts of samples increased significantly from 6.0-8.0 log CFU/g on day 0 (after stuffing) up to 8.7-10.9 log CFU/g on day 3 (end-fermentation), and then decreased to 4.6-8.5 log CFU/g on day 14 (end-ripening). Further numerically reduction in LAB counts was also observed in process II samples on day 28 (post-ripening storage). After day 3 (end-fermentation), the TPC counts of process II samples were significantly higher than that of process I samples. This difference in TPC counts between samples produced by the two processing methods was more obvious on day 14 (i.e. end-ripening). Similarly, except for some minor exceptions, the LAB counts of process II samples were higher than that of process I samples after day 3.

After fermentation, lactic acid bacteria became predominant, and had been reported in many types of fermented sausages (Roig-Sagués *et al*., 1999; Rubio *et al*., 2007).

Pediococcus cerevisiae and *Lactobacillus plantarum* were found to be predominant in the fermentation stage of mum sausages manufactured (Somathiti and Suraphanthaphisit, 1987). *Pediococcus acidilactici, L. plantarum 1, L. plantarum 2, and Lactobacillus cellobiosus* were the major microorganisms isolated from the mums that were purchased from local markets in Chiang Mai, Chaiyapoom, and Khon Kaen of Thailand (Wiriyacharee *et al*., 2003). Rubio *et al*. (2007) explained that this predominance of lactic acid bacteria was probably due to the good adaptation to the meat environment and faster growth rates of microorganisms during sausage fermentation and ripening. In this study, a similar microbial change pattern was observed between the total microorganisms and lactic acid bacteria during processing, and was in agreement with Roig-Sagués *et al*. (1999) who reported that a statistically significant correlation (R^2=0.99) was observed between the LAB and aerobic mesophilic bacteria counts in Spanish ripened sausages.

In this study, the initial *Enterobacteriaceae* counts in sausage ranged from 4.8-6.6 log CFU/g on day 0 (after stuffing) and decreased to 2.9-5.0 log CFU/g on day 3 (end-fermentation), and 0-2.7 log CFU/g on day 14 (end-ripening). No *Enterobacteriaceae* was detectable for process II samples on day 28 (post-ripening storage). The enterobacteria counts of Spanish sausages (fuet) decreased steadily during ripening and were undetectable after ripening for 12 days at 15–18°C with 67–82% relative humidity and post-ripening storage at room conditions for 22 days (Roig-Sagués *et al*., 1999). The authors indicated that some factors, such as a low initial pH value, a low initial water activity, a high concentration of fermentable carbohydrates, high numbers of *lactobacilli* in the fresh sausage mixture, the presence of nitrate or nitrite, and low enough ripening temperatures, did not favor the growth of *Enterobacteriaceae*. González and Díez **(**2002) indicated that due to the high sensitivity to acidity and desiccation, *Enterobacteriaceae* are rarely found in long-ripening meat products. Additionally, inoculation of a starter culture (*Lactobacillus sake* CL35) when making dry fermented Spanish sausages resulted in *Enterobacteriaceae* undetectable after 12 days while the *Enterobacteriaceae* did not disappear in the control sausages until day 24 (González and Díez, 2002). The authors explained that the antimicrobial effect was partially due to the antibiotic-like substances excreted by *L. sakei*. In the same study, a more significant reduction in microbial counts was observed when the

product contained nitrite (50-100 ppm) and was inoculated with a starter culture during processing. In this study, the fermentation promoted the growth of lactic acid bacteria and further caused the disappearance of *Enterobacteriaceae* within a few days, and agreed with the report by González and Diez (2002).

4.4. Texture and sensory characteristics

The results of texture profile analysis are presented in Table 9. The results showed that the hardness of all samples increased from day 0 (after stuffing) to day 3 (end-fermentation). A continuous and more significant increase in hardness was observed in process I samples, whereas the hardness of process II samples remained stable until day 28 (post-ripening storage). Similar change patterns were also observed in chewiness and adhesiveness of samples. Due to the faster dehydration in process I samples which were stored at 30°C without any packaging, the hardness and chewiness of sausages increased significantly to 125-198 N and 15-28 N mm, respectively on day 14 (end-ripening process). Springiness and cohesiveness of all samples decreased significantly on day 3. Except for some minor exceptions, continuous and more significant decreases in springiness and cohesiveness were observed in process I samples, whereas these two characteristics of process II samples remained stable until day 28 (post-ripening storage). Process I sausages had too tough texture and was not acceptable for sensory tests during further storage. On the other hand, stable and consistent texture characteristics made process II samples still acceptable up to day 28. The results have demonstrated that process II samples, which were vacuum-packaged and stored at 4°C until day 28, had more consistent and less change in texture characteristics and an extended shelf-life.

During the fermentation process, the pH gradually declined due to the accumulation of lactic acid which induced protein aggregations and led to the formation of an ordered protein network, and finally contributed to the firmness of products (González-Fernández *et al*., 2006). Additionally, after fermentation, drying also influenced the texture properties of final products. In this study, because process I samples had unacceptable toughness on day 14 (end-ripening), only process II samples were evaluated for the sensory characteristics (Figure 10). No significant differences ($P > 0.05$) in color, flavor, texture, and overall acceptance of process II samples were detected between day 3 and day 28. Influences in the

lipid oxidation and color stability of dry fermented sausage during chilled storage due to vacuum-packaging were also reported (Rubio *et al*., 2008). The mum sausage from Chaiyaphum province had higher scores in all sensory parameters than the ones from Khon Kaen province and it was probably due to different manufacturing formulas and spices and seasonings used.

Many fermented sausages which produced all over the world have their unique characteristics (Ordóñez *et al*., 1999). For example, some European-style sausages are fermented and dried at lower temperature (approximately below 25℃) for 3 to 7 weeks (Vignolo *et al*., 2010), whereas the US-style fermented sausages are frequently fermented and dried at higher temperatures (approximately above 35℃) for a shorter period of time (Ordóñez *et al*., 1999; Vignolo *et al*., 2010). In Thailand and other Asian countries, on the other hand, sausages are normally fermented naturally (i.e. without adding starter cultures during manufacturing) at an ambient temperature for approximately 2-3 days (Chockchaisawasdee *et al*., 2010; Sriphochanart and Skolpap, 2011). Not like those European- and US-style sausages which are normally eaten after fermentation and ripening process, Thai fermented sausages are commonly consumed immediately after fermentation either with a comparatively shorter time of ripening or sometime even without through a ripening process (Yodrak, 2003; Rotsatchakul *et al*., 2009; Chockchaisawasdee *et al*., 2010).

Many intrinsic and extrinsic factors, such as temperature, water activity, acidity, redox potential, preservative microflora, and etc., are considered as "hurdles" which influence the fates of microorganisms in products and eventually the shelf-life of products (Leistner, 2000). In most cases, shelf lives of Thai fermented sausages are approximately 10-15 days after stuffing depending on the products, processing and storage conditions (Yodrak, 2003), which were much shorter than the one of European and US-style ones (Rubio *et al*., 2007). Currently, most fermented sausages produced in Thailand are manufactured conventionally. Therefore, the qualities and shelf lives of products vary a lot. Additionally, too intensive toughness and hardness of the process I sausages in this study were probably due to a high ambient temperature which the sausages were fermented and stored (i.e. approximately 30°C in this study), and a faster drying rate at this condition, thus

it led a pronounced surface protein coagulation of samples and eventually made them not acceptable by consumers after storage for 14 days (Andrés *et al*., 2007).

Table 6 Changes in proximate composition and water activity of mum sausages during processing

Treatment	Processing time (day)			
	0	3	14	28
Moisture (%)				
CP-I	72.36±0.12 ^{a,A}	52.35±0.08 ^{b,B}	38.82±0.21 ^{c,B}	—
CP-II	71.14±0.13 ^{a,B}	54.62±0.22 ^{b,A}	52.08±0.07 ^{c,A}	50.21±0.02 ^{d}
KK-I	69.89±0.11 ^{a,A}	52.35±0.07 ^{b,B}	39.76±0.20 ^{c,B}	—
KK-II	70.41±0.28 ^{a,A}	55.08±0.52 ^{b,A}	53.58±0.34 ^{c,A}	51.14±0.53 ^{d}
Protein (%)				
CP-I	20.61±0.35 ^{c,A}	33.83±0.23 ^{b,A}	40.66±0.29 ^{a,A}	—
CP-II	18.61±0.77 ^{c,B}	30.12±0.71 ^{b,B}	32.54±0.15 ^{a,B}	33.99±0.33 ^{a}
KK-I	18.46±0.14 ^{c,B}	29.66±0.07 ^{b,B}	40.37±0.12 ^{a,A}	—
KK-II	20.39±0.00 ^{d,A}	32.89±0.00 ^{c,A}	35.67±0.00 ^{b,B}	36.91±0.00 ^{a}
Fat (%)				
CP-I	2.44±0.03 ^{c,A}	4.19±0.01 ^{b,A}	4.81±0.04 ^{a,A}	—
CP-II	2.35±0.01 ^{d,B}	3.98±0.02 ^{c,B}	4.21±0.02 ^{b,B}	4.35±0.02 ^{a}
KK-I	3.14±0.00 ^{c,A}	4.43±0.02 ^{b,A}	5.46±0.00 ^{a,A}	—
KK-II	3.09±0.00 ^{d,B}	4.37±0.01 ^{c,B}	4.49±0.01 ^{b,B}	4.60±0.01 ^{a}
Ash (%)				
CP-I	3.49±0.01 ^{c,A}	4.46±0.02 ^{b,A}	4.92±0.05 ^{a,A}	—
CP-II	3.50±0.00 ^{d,A}	4.16±0.01 ^{c,B}	4.35±0.00 ^{b,B}	4.49±0.01 ^{a}
KK-I	2.88±0.03 ^{c,A}	3.80±0.01 ^{b,B}	4.65±0.02 ^{a,A}	—
KK-II	2.66±0.02 ^{d,B}	3.99±0.01 ^{c,A}	4.11±0.00 ^{b,B}	4.27±0.02 ^{a}
Water activity				
CP-I	0.983±0.001 ^{a,A}	0.959±0.001 ^{b,A}	0.823±0.001 ^{c,B}	—
CP-II	0.980±0.000 ^{a,A}	0.942±0.002 $^{b.B}$	0.937±0.001 ^{c,A}	0.928±0.001 ^{d}
KK-I	0.983±0.001 ^{a,A}	0.955±0.002 ^{b,A}	0.894±0.001 ^{c,B}	—
KK-II	0.984±0.002 ^{a,A}	0.944±0.002 $^{b.B}$	0.932±0.001 ^{c,A}	0.927±0.001 ^{c}

—: not determined.

$^{a–d}$ Means within the same row with different superscripts are significantly different ($P < 0.05$).

$^{A–B}$ Means within the same column for the same test and products from the same provinces with different superscripts are significantly different ($P < 0.05$).

CP and KK: mum sausages collected from Chaiyaphum and Khon Kaen provinces, respectively. Process I: freshly-manufactured sausages stored at ~30°C until day 14; process II: freshly-manufactured sausages stored at ~30°C until day 3, vacuum-packaged, and then stored at 4°C until day 28.

Table 7 Changes in pH value and titratable acidity of mum sausages during processing

Treatment	Processing time (day)			
	0	3	14	28
pH value				
CP-I	5.18±0.01 ^{a,B}	4.41±0.01 ^{c,A}	4.53±0.01 ^{b,A}	—
CP-II	5.29±0.00 ^{a,A}	4.39±0.01 ^{d,A}	4.42±0.01 ^{c,B}	4.50±0.01 ^{b}
KK-I	5.34±0.02 ^{a,B}	5.10±0.00 ^{c,A}	5.25±0.01 ^{b,A}	—
KK-II	5.46±0.00 ^{a,A}	4.86±0.01 ^{b,B}	4.68±0.01 ^{c,B}	4.66±0.01 ^{d}
Titratable acidity (%)				
CP-I	0.53±0.01 ^{b,B}	2.51±0.01 ^{a,A}	2.53±0.01 ^{a,A}	—
CP-II	0.85±0.01 ^{c,A}	2.11±0.01 ^{b,B}	2.14±0.00 ^{a,B}	2.09±0.01 ^{b}
KK-I	0.74±0.04 ^{c,A}	1.48±0.00 ^{b,B}	1.74±0.01 ^{a,A}	—
KK-II	0.63±0.01 ^{c,A}	1.64±0.01 ^{a,A}	1.59±0.01 ^{b,B}	1.57±0.01 ^{b}

—: not determined.

$^{a–d}$ Means within the same row with different superscripts are significantly different ($P < 0.05$).

$^{A–B}$ Means within the same column for the same test and products from the same provinces with different superscripts are significantly different ($P < 0.05$).

CP, KK, Process I, and II: abbreviations are the same as Table 6.

Table 8 Changes in color parameters of mum sausages during processing

Treatment	Processing time (day)			
	0	3	14	28
L* value				
CP-I	45.99±0.16 ^{c,A}	47.67±0.38 ^{b,A}	49.12±0.60 ^{a,A}	—
CP-II	44.45±0.17 ^{c,B}	48.56±0.33 ^{b,A}	49.24±0.36 ^{ab,A}	49.98±0.57 ^{a}
KK-I	41.49±0.26 ^{c,A}	45.92±0.06 ^{b,B}	48.26±0.17 ^{a,A}	—
KK-II	40.38±0.30 ^{b,B}	49.14±0.12 ^{a,A}	48.12±0.55 ^{a,A}	48.67±0.53 ^{a}
a*value				
CP-I	10.24±0.30 ^{a,A}	8.94±0.34 ^{b,A}	7.88±0.21 ^{c,A}	—
CP-II	9.14±0.21 ^{a,B}	7.48±0.13 ^{b,B}	6.85±0.24 ^{c,B}	6.94±0.1 ^{bc}
KK-I	6.72±0.16 ^{a,A}	5.64±0.33 ^{b,A}	4.76±0.15 ^{c,B}	—
KK-II	6.61±0.35 ^{a,A}	5.37±0.26 ^{b,A}	5.46±0.20 ^{b,A}	5.98±0.38 ^{ab}
b*value				
CP-I	6.02±0.08 ^{c,B}	8.38±0.21 ^{b,B}	9.07±0.09 ^{a,B}	—
CP-II	7.48±0.23 ^{c,A}	10.48±0.12 ^{a,A}	9.43±0.07 ^{b,A}	10.13±0.09 ^{a}
KK-I	6.52±0.30 ^{c,B}	8.19±0.12 ^{b,B}	9.89±0.18 ^{a,A}	—
KK-II	6.40±0.18 ^{d,B}	9.13±0.22 ^{c,A}	9.88±0.23 ^{b,A}	10.67±0.27 ^{a}

—: not determined.

$^{a–d}$ Means within the same row with different superscripts are significantly different ($P < 0.05$).

$^{A–B}$ Means within the same column for the same test and products from the same provinces with different superscripts are significantly different ($P < 0.05$).

CP, KK, Process I, and II: abbreviations are the same as Table 6.

Table 9 Changes in texture profiles of mum sausages during processing

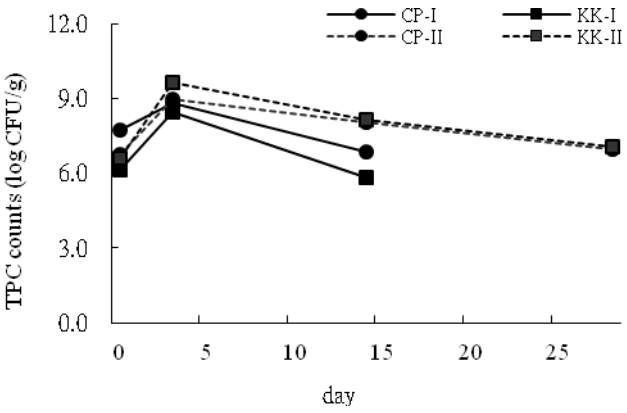
Treatment	Processing time (day)							
	0		3		14		28	
Hardness (N)								
CP-I	7.35±0.34	c,A	33.46±0.73	b,B	125.48±0.6	a,A	—	
CP-II	8.32±0.40	b,A	39.66±1.13	a,A	39.65±0.90	a,B	40.87±1.21	a
KK-I	8.24±0.37	c,A	42.09±0.74	b,B	197.65±3.98	a,A	—	
KK-II	7.29±0.26	b,A	48.97±0.81	a,A	47.25±0.52	a,B	48.67±0.93	a
Springiness (mm)								
CP-I	0.91±0.06	a,A	0.54±0.04	b,A	0.43±0.03	b,A	—	
CP-II	0.84±0.09	a,A	0.57±0.07	b,A	0.59±0.09	b,A	0.54±0.07	b
KK-I	0.87±0.04	a,A	0.55±0.03	b,A	0.49±0.03	b,B	—	
KK-II	0.78±0.04	a,A	0.59±0.03	b,A	0.61±0.05	b,A	0.55±0.04	b
Cohesiveness (ratio)								
CP-I	0.57±0.05	a,A	0.36±0.05	b,A	0.27±0.04	c,A	—	
CP-II	0.54±0.07	a,A	0.52±0.07	a,A	0.39±0.07	a,A	0.40±0.03	a
KK-I	0.54±0.01	a,A	0.37±0.02	b,A	0.29±0.01	c,B	—	
KK-II	0.51±0.03	a,A	0.41±0.02	b.A	0.42±0.02	b,A	0.38±0.03	b
Chewiness (N mm)								
CP-I	3.83±0.39	b,A	6.58±1.33	b,B	15.07±2.87	a,A	—	
CP-II	3.56±0.27	b,A	11.61±1.75	a,A	8.63±1.79	a,B	8.69±1.23	a
KK-I	3.86±0.35	c,A	8.55±0.50	b,B	27.98±2.01	a,A	—	
KK-II	2.92±0.32	b,A	11.75±1.1	a,A	12.09±0.24	a,B	9.97±0.83	a
Adhisiveness (Ns)								
CP-I	-0.43±0.06	b,A	-0.28±0.06	b,A	-0.05±0.01	a,A	—	
CP-II	-0.41±0.06	b,A	-0.25±0.05	a,A	-0.23±0.05	a,B	-0.26±0.03	a
KK-I	-0.35±0.08	b,A	-0.32±0.04	b,A	-0.04±0.01	a,A	—	
KK-II	-0.31±0.09	a,A	-0.19±0.04	a,A	-0.15±0.03	a,B	-0.16±0.03	a

—: not determined.

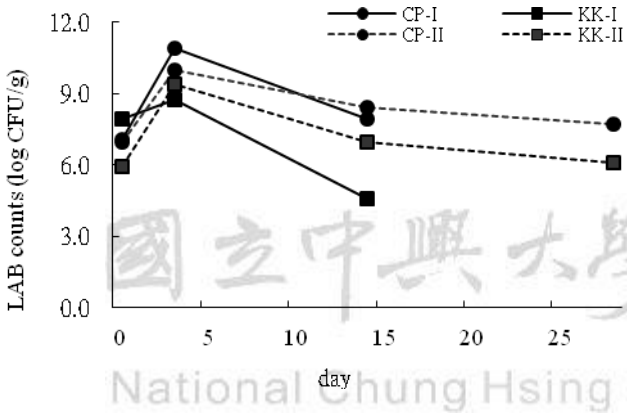
[a–d] Means within the same row with different superscripts are significantly different ($P < 0.05$).

[A–B] Means within the same column for the same test and products from the same provinces with different superscripts are significantly different ($P < 0.05$).

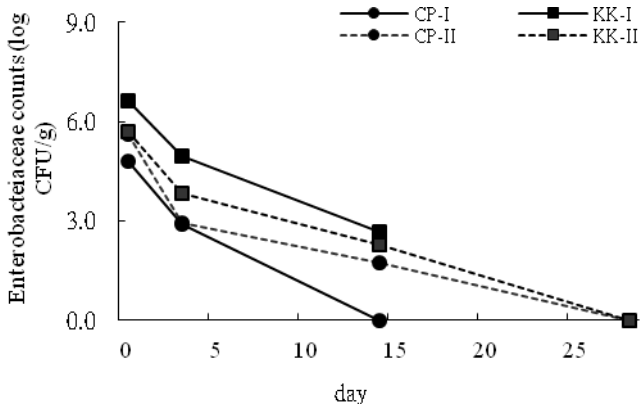
CP, KK, Process I, and II: abbreviations are the same as Table 6.



(a)

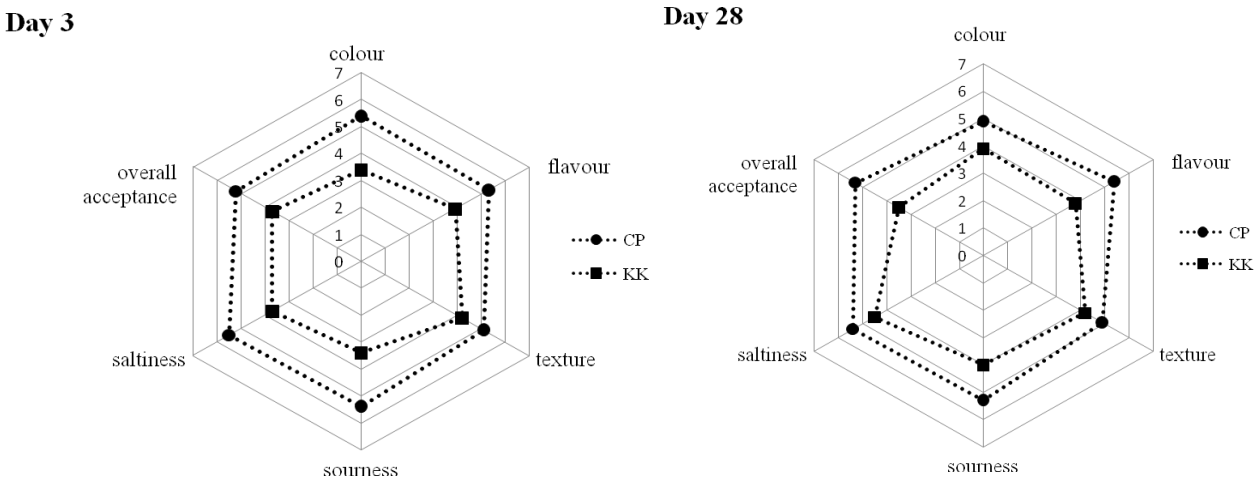


(b)



(c)

Figure 9. Changes in the counts of (a) total microflora, (b) lactic acid bacteria, and (c) *Enterobacteriaceace* of mum sausages during processing.
Abbreviations are the same as Table 6.



(a) (b)

Figure 10. Sensory evaluation of process II mum sausages (a) at day 3 (end-fermentation) and (b) at day 28 (post-ripening storage).
Abbreviations are the same as Table 6.

CHAPTER 4

Monitoring the dynamics of lactic acid bacteria population during manufacturing and storage of mum (Thai fermented sausage) based on restriction fragment length polymorphism (RFLP) analysis

1. Abstract

The aim of this study was to apply restriction fragment length polymorphism (RFLP) for identifying the lactic acid bacteria (LAB) that were isolated from mum sausages during manufacturing and storage. LAB were isolated from the sausages during manufacturing by two methods (method I: after stuffing, sausages were fermented and stored at ~30°C for 14 days; method II: after stuffing, sausages were fermented at ~30°C for three days, then were vacuum-packaged and stored at 4°C until day 28). The 16S rDNA of LAB isolate was amplified by polymerase chain reactions and digested with restriction enzymes. The results showed that *Dde* I presented the highest discrimination capacity. LAB were classified and further identified as *Lactobacillus sakei, Lactobacillus plantarum, Leuconostoc mesenteroides, Lactobacillus brevis, Lactobacillus fermentum, Pediococcus pentosaceus*, and *Lactococcus lactis*. During fermentation and storage, *L. sakei* and *L. plantarum* were dominant in both methods. For method II, the proportion of *Ln. mesenteroides* increased remarkably during post-ripening storage and became predominant similar to *L. sakei.* The identification of LAB in samples helps to select appropriate microorganisms as candidate starter cultures for future use.

Keywords: fermented sausage, lactic acid bacteria, mum sausage, RFLP

2. Introduction

Mum, which is a traditional Thai fermented sausage, is commonly produced and consumed in the north-eastern region of Thailand. Some ingredients, including beef, liver, spleen, ground roasted rice, salt, and garlic, are mixed and stuffed into a natural casing and then held at room temperature for two or three days until a final acceptable sour-tasting flavor is developed (Thai Industrial Standards Institute, 2003). Traditionally, mum sausage is produced by utilizing the natural lactic acid bacteria (LAB), and it often results in products with inconsistent qualities. Furthermore, such natural microflora often contain some beneficial microorganisms for the development of fermentation flavor and texture of products, but they may also include some spoilage species or even pathogenic microorganisms (Talon *et al*., 2007). The specific characteristics and qualities of the final

products are mainly influenced by the raw materials used, the manufacturing techniques and the agro-ecosystem of the area of production (Albano *et al*., 2007). Understanding the microbial ecology of fermented products is critical in order to evaluate the physicochemical and sensory changes during fermentation and maturation (Comi *et al*., 2005).

Lactobacilli, *Pediococci*, and *Micrococci* are the most commonly bacteria found in Thai fermented meat products, yet the precise role of these bacteria in the product quality is not fully understand (Thiravattanamontri *et al*., 1998). Producers are interested in improving the qualities of products and also extending the shelf-life of products in order to satisfy consumers' demands and possibly increase the potential market. Some unique properties, such as superior abilities of proteolysis, flavor development, pH decrease, and etc., cause *L. sakei* and *L. plantarum* frequently to be applied as starter cultures during manufacturing of fermented products (Sriphochanart and Skolpap, 2010). Being better adapted to the complicated fermentation environment, Lee *et al*. (2006) suggested that *L. plantarum*, which was isolated from kimchi, could be a potential starter culture in sausage production.

Due to the important role of LAB in food fermentation, research to define and develop starter cultures of LAB would enable a more controllable process and ensure a greater consistency in quality and safety of products for consumers. The identification of these microorganisms is of great scientific and practical interest and importance. Research applying some molecular techniques based on the DNA or RNA sequence has been conducted for microbial identification with accuracy and efficiency. Polymerase chain reaction linked with restriction fragment length polymorphism analyses (PCR-RFLP) has been applied to differentiate LAB in several studies (Chenoll *et al*., 2004; Claisse *et al*., 2007; Yanagida *et al*., 2008). Therefore, this study aimed (1) to identify the dominant LAB species and to evaluate the variation in the bacterial community composition of LAB in mum that manufacturing with the conventional processing (fermented and stored at ~30°C until day 14) or a modified processing (fermented at ~30°C until day 3, vacuum-packaged, and then stored at 4°C until day 28), and (2) to evaluate the feasibility and efficiencies of restriction enzymes to differentiate lactic acid bacteria in restriction fragment length fragment polymorphism (RFLP) analysis.

3. Materials and methods

3.1. Sausage preparation and sampling

Sausage preparation was conducted by the method of Wanangkarn *et al*., (2012). Freshly-manufactured mum sausages were collected from a local meat factory (Chaiyaphum, Thailand), transported to the lab, and assigned to the two processing methods. For method I, which represented the conventional method, after collection, sausages were hung vertically on stainless steel hangers under an environment of 30 ± 2℃ and 65 ± 2% relative humidity until day 14 without any packaging. For method II which represented a modified method, after collection, sausages were hung vertically on stainless steel hangers at 30 ± 2℃ and 65 ± 2% relative humidity until day 3. They were then vacuum-packaged in a vacuum bag (laminate of Nylon/LLDPE, Chun I Gravure Co., Ltd., Taichung, Taiwan) and stored at 4°C until day 28. Sausages were sampled on day 0 (after stuffing), day 3 (end-fermentation), day 14 (end-ripening process), and day 28 (post-ripening storage) for analyses.

3.2. Isolation of lactic acid bacteria

A 25-g sample was aseptically placed in a sterile bag, which contained 225 ml of 0.85% NaCl solution, and homogenized with a stomacher (Oskon Co., Ltd., Thailand) for 2 min. Serial dilutions were made and poured into Man Rogosa Sharpe (MRS) agar (Merck, Dram Stadt, Germany), and incubated at 37℃ for 48 h in an anaerobic jar (BBL GasPak System, USA) (APHA, 2001). At each sampling time (method I: day 0, 3, and 14; method II: day 0, 3, 14, and 28), thirty colonies from each plate (sampling time/method) were randomly selected by using the Harrison's disc method (Harrigan, 1998) and purified by successively streaking on MRS agar plates. The selected colonies were transferred to MRS broth and stored as liquid cultures with 30% (v/v) glycerol as a cryoprotectant at -80℃ before being subjected to molecular identification (Papamanoli *et al*., 2003).

3.3. Extraction and preparation of genomic DNA from LAB isolates

LAB isolates were activated in MRS broth at 30℃ for 12 h before analysis, and then genomic DNA of the isolates was extracted using a GF-1 bacterial DNA extraction kit (Vivantis Technologies, Selangor Darul Ehsan, Malaysia) according to the manufacturer's instructions. The extracted genomic DNA from each LAB isolate was checked by

horizontal gel electrophoresis (Bio-RAD, California, USA) with 1.5% (w/v) agarose containing 0.5 µg/ml ethidium bromide in 1X TBE using 100 V for 20 min. The gel was visualised and photographed via ultraviolet transilluminator (ChemiDoc™ MP System, Bio-RAD, California, USA). The DNA concentration and purity were also determined using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, Delaware, USA). The DNA preparations were stored at -20°C until required.

3.4. Amplification of 16S rDNA of LAB isolates

DNA from the -20°C stock was diluted to 100 ng/µl with nuclease-free double distilled water. The 16S rDNA fragment was amplified by polymerase chain reactions (PCR) with an universal primer set of BSF8/20 (5'-AGAGTTTGATCCTGGCTCAG-3') as a forward primer and REVB (5'-GGTTACCTTGTTACGACTT-3') as a reverse primer (Weisburg *et al*., 1991) using a PTC-200 Thermo Cycler (MJ Research Inc., Watertown, MA, USA). All reagents used in PCR amplification were purchased from Fermentas International Inc. (Ontario, Canada). The amplification was done in 50 µl reaction volumes, and each PCR reaction consisted of 250 µM of each dNTP, 10 µM of each primer (BioDesign Co., Ltd., Pathumthani, Thailand), 50 mM of $MgCl_2$, 0.5 µl of 10X PCR buffer, 5 U of Taq polymerase, and 5 µl of template DNA. The thermal cycling including an initial denaturation step at 95°C for 3 min; 35 cycles of denaturation step at 94°C for 1 min, an annealing step at 50°C for 1 min, an extension step at 72°C for 2 min; and a final extension at 72°C for 10 min. The PCR product was checked using 1.5% (w/v) agarose gel electrophoresis. The gel was visualised and photographed via an ultraviolet transilluminator.

3.5. Restriction analyses of 16S rDNA PCR products

The 16S rDNA PCR product from each LAB isolate was digested with *Dde* I (C/TNAG), *Alu* I (AG/CT), *Mse* I (TT/AA) or *Aci* I (AA/CGTT) according to Bonomo *et al.* (2008), Claisse *et al.* (2007), and Rodas *et al.* (2003). Following the procedures described by Fermentas International Inc. (Ontario, Canada), the restriction endonuclease reaction mixture was mixed gently and spun down for a few seconds. The reaction mixture was then incubated at 37°C for 3 h, and the restriction patterns were examined by using 2% (w/v) agarose gels in 1X TBE buffer at 100 V for 45 min [horizontal gel electrophoresis (Bio-RAD, California, USA)] with a DNA ladder (GeneRuler^{TM} 100 bp DNA ladder). The gel

was visualised and photographed by an UV transilluminator (ChemiDoc™ MP System, Bio-RAD, California, USA).

3.6. 16S rDNA sequencing

Representative of each RFLP pattern group was randomly chosen for sequence analysis. Prior to sequencing, the 16S rDNA was purified by QIAquick PCR Purification Kit/250 (QIAGEN GmbH, Hilden, Germany). The same primers used in the amplification steps were utilized for the sequencing of both strands of the PCR products, respectively. Sequencing reactions were analyzed with a MegaBACE 1000 sequencer (BioDesign Co., Ltd., Pathumthani, Thailand). The identities of these isolates were determined through a search of the GenBank DNA database (http://www.ncbi.nlm.nih.gov/BLAST) by using the basic local alignment search tool (BLAST). Phylogenetic and molecular evolutionary analyses were conducted with the MEGA software by using the neighbour-joining method and kimura two parameter model with 1000 bootstrap replicates to construct distance-based trees (Antara *et al*., 2002).

4. Results and discussion

4.1. Extraction of genomic DNA and amplification of 16S rDNA of LAB isolates

In this study, LAB isolates were randomly selected from agar plates and subjected to molecular identification, and 16S rDNA amplification fragment was obtain from DNA templates as presented in the Figure 11. Extraction of genomic DNA from the LAB isolates was accomplished by using commercially available DNA extraction kits (Atshan and Shamsudin, 2011). All DNA samples were then applied for the PCR amplification by using universal BSF8/20 and REVB primers for amplifying 16S rDNA. The Fig. 11b revealed a PCR amplification product with ~1,500 bp, and agreed with other studies (Rattanachomsri *et al*., 2011; Shukla and Goyal, 2011; Singh and Ramesh, 2009).

4.2. Restriction analyses of 16S rDNA PCR products

The 16S rDNA amplification fragment of LAB isolates was classified by using an individual restriction enzyme of *Aci* I, *Alu* I, *Mse* I or *Dde* I in restriction fragment length polymorphism (RFLP) analysis. In this study, *Aci* I restriction enzyme, which did not cleave 16s rDNA into small fragments, was not able to distinguish the LAB that isolated

from the mum sausages (Fig. 12a), similarly to the study of Hsieh *et al*. (2010) found that *Aci* I did not cleave PCR products but functioned well when applied to differentiate bacteria that isolated from dry-dressed fish fillets. Additionally, Claisse *et al.* (2007) reported that *Lactobacillus* species couldn't be distinguished from the other LAB rods when digested with a single *Aci* I enzyme. The ineffectiveness of *Aci* I might be also related to its recognition sequence (AA/CGTT), which was more specific by having 6 nucleotides when comparing to the other restriction enzymes that have less nucleotides (i.e. 4, 4, and 4 nucleotides of *Alu* I, *Mse* I, and *Dde* I, respectively) (Bonomo *et al*., 2008; Claisse *et al*., 2007).

Rodas *et al*. (2003) reported that *L. plantarum* and *P. pentosaceus* could be discriminated by *Alu* I. In this study, totally five restriction patterns were observed when digested with *Alu* I (Fig 12b). Among them, four profiles (lanes A, B, D, and F which could be identified as *L. plantarum, L. sakei, L. brevis*, and *Ln. mesenteroides*, respectively, by using sequence analysis) could be distinguished. However, the restriction profiles of lanes C, E, and G (*L. fermentum, P. pentosaceus,* and *Lc. lactis*, respectively) were not able to be distinguished. *Alu* I has been applied to identify *Lactobacillus* spp. when developing a rapid diagnostic assay in *Lactobacillus bacteremia* diagnosis (Christensen *et al*., 2004). When isolating and characterizing LAB from koshu vineyards in Japan, Yanagida *et al*. (2008) reported that by using 16S rDNA RFLP and digestion with *Alu* I, it was possible to discriminate between *L. plantarum, Lc. lactis*, and *P. pentosaceus.* In this study, a different *Alu* I digested pattern of *L. plantarum* (lane A) could be differentiated with those of *P. pentosaceus* (lane E) and *Lc. lactis* (lane G); however, the restriction patterns between *P. pentosaceus* (lane E) and *Lc. lactis* (lane G) was not distinguishable. The differences between our and other studies were possibly due to the different strains evaluated. Additionally, Mainville *et al.* (2006) suggested that a very small difference in a genetic locus might not be detectable by RFLP analysis, thus leading to a limited discrimination between the LAB strains evaluated.

In this study, the 16s rDNA amplified products of LAB that were digested with *Mse* I generated 7 characteristic profiles (Fig. 12c). However, the digestion had some disadvantages because of appearing some ambiguous bands on the gels. Rodas *et al*. (2003)

reported that *Mse* I showed a high discrimination capacity to differentiate *Lactobacillu*s and *Pediococcus* genus. Comparing with *Alu* I, which was not able to distinguish *L. fermentum*, *P. pentosaceus*, and *Lc. lactis* in this study (Fig 12b), the *Dde* I showed a superior discrimination capacity to other enzymes applied (Fig 12d). It was able to distinguish the seven LAB isolates from the mum sausages, and these LAB could be identified later as *L. sakei, L. plantarum, Ln. mesenteroides, L. brevis, L. fermentum, P. pentosaceus*, and *Lc lactis* as shown in Table 10. Similarly, Bonomo *et al*. (2008) reported that *Dde* I was able to distinguish 6 LAB, which was isolated from the traditional Italian fermented sausages. Among these 6 LAB, *L. sakei*, *L. plantarum*, *L. brevis*, and *P. pentosaceus* were also isolated from the mum sausages. In a study done by Chenoll *et al*. (2003), ARDRA-*Dde* I presented the best discrimination ability among the species of genus *Lactobacillus*, and *Pediococcus* could be achieved by restriction of ISR with *Dde* I.

4.3. Identification by sequencing of the 16S rDNA gene

In this study, seven representative RFLP band patterns from *Dde* I digestion were selected for further sequencing. The sequences of 16S rDNA of LAB were aligned for their identity and compared to the DNA sequences in the GenBank database (http://www.ncbi.nlm.nih.gov) by using the BLAST algorithm. Table 10 shows the identity percentage of the LAB species in relation to those illustrated in the database and provides GenBank accession number. In this study, seven LAB species isolated from the mum sausages and digested with *Dde* I restriction enzyme were identified as *L. plantarum, L. sakei, L. fermentum, L. brevis, P. pentosaceus, Ln mesenteroides*, and *Lc. lactis* (Table 10). Some of them are commonly found in a variety of Thai fermented foods (Tanasupawat, 2009). Also, results of the 16S rDNA sequences indicated that lanes A, B, C, D, E, F and G were closely positioned to *L. plantarum* (98% identity), *L. sakei* (98% identity), *L. fermentum* (98% identity), *L. brevis* (98% identity), *P. pentosaceus* (97% identity), *Ln mesenteroides* (99% identity), and *Lc. lactis* (99% identity), respectively.

Alignment of 16S rDNA sequences of representative and reference strains determined their phylogenetic relationship by using the neighbor-joining method and kimura two-parameter model (Fig. 13). As mentioned by Ennahar *et al*. (2003), phylogenetic trees based on the 16S rDNA sequence, displayed high consistency regarding

the relationships between the organisms included. In this study, the 16s rDNA sequences tree shows that two distinct groups of LAB: *Ln. mesenteroides* alone and all the other species. In the sequence phylogenetic tree, the lanes of A, B, C, D, E, F, and G were clearly assigned to the species of *L. plantarum, L. sakei, L. fermentum, L. brevis, P. pentosaceus, Ln. mesenteroides*, and *Lc. lactis* with a 100% bootstrap cluster, respectively.

4.4. LAB population dynamics during manufacturing and storage of mum

The genus of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Lactococcus* were identified as the major LAB found in dry fermented sausages (Adiguzel and Atasever, 2009; Danilović *et al*., 2011; Fontana *et al*., 2005). Thiravattanamontri *et al.* (1998) also indicated that *Lactobacilli* and *Pediococci* were commonly detected in Thai fermented foods. *Lactobacillus* was the most common LAB in this study and the dominant flora in other dry fermented sausages (Adiguzel and Atasever, 2009).

Figures 14a and 14b illustrate that the LAB dynamics for method I (the conventional method) and II (the modified method) samples during various stages, respectively. The majority of LAB isolated from day 0 and day 3 samples were assigned to the species of *L. plantarum* and *L. sakei* in both method samples. At day 3, the percentage of *L. sakei, L. fermentum*, and *Lc. lactis* increased whereas the percentages of *L. brevis, P. pentosaceus,* and *Ln. mesenteroides* decreased in method I samples. At day 14, the microorganisms were presented in ascending order of *L. sakei, L. plantarum,* and *L. fermentum,* whereas small amount of *Lc. lactis* and no *Ln. mesenteroides, P. pentosaceus, L. brevis* were observed in method I samples. This result agreed with Lee *et al.* (2006) who reported that *Lactococcus* and *Pediococcus* increased initially and then decreased in the ripening process (at temperatures of 20-30°C). For method II samples, *Ln. mesenteroides* increased in proportion remarkably and became dominant, followed by *L. sakei, L. brevis*, and *Lc. Lactis*, and no *L. plantarum* and *L. fermentum* were detected at day 14 and day 28. According to Lee *et al.* (2006) and Wu *et al.* (2009), *Lactobacilli* species could continuously grow throughout the fermentation process, and this was possible because *Lactobacilli* were more acid tolerant than other species.

Lee *et al*. (2006) reported that temperature and fermentation development played important roles in influencing the growth of LAB. The ability of *L. plantarum* and *L. sakei*

to grow under various conditions has been reported. Papamanoli *et al.* (2003) indicated that the optimum temperature of *L. plantarum* was ranges from 15°C to 45°C and might not grow at 4°C. On the other hand, *L. sakei* was capable of growing in a widely temperature range of 4-45°C, and was reported to become predominant in vacuum-packaged or unpacked sausages (Danilović *et al.*, 2011). This might be the reason why after day 14, only *L. sakei,* but not *L. plantarum,* were detected in method II (4°C and anaerobic) samples. *L. fermentum* could not growth at temperature lower than 10°C and requiring some oxygen for maintaining a fermentative metabolism, which was reported by Suutari and Laakso (1992), possibly made *L. fermentum* not detectable in method II samples after day 14.

Small amounts of *Ln. mesenteroides* were detected in method I and II samples during fermentation, whereas its percentage increased remarkably and became predominant in method II samples after day 14. This was possible because *Ln. mesenteroides* were able to grow at 4°C, but not above 30°C, and preferred an anaerobic condition (Cai *et al*., 1998; Danilović *et al*., 2011). *Ln. mesenteroides,* which was previously isolated from vacuum-packaged sliced ham, has been implicated with some negative influence on the sensory characteristics of products probably due to its acid production (Cai *et al*., 1998). However, Lee *et al*. (2006) indicated that in addition to lactic acid, *Ln. mesenteroides* produced other metabolites, such as acetic acid, acetaldehyde, diacetyl, and ethanol, and eventually enhanced the flavor development in fermented sausages.

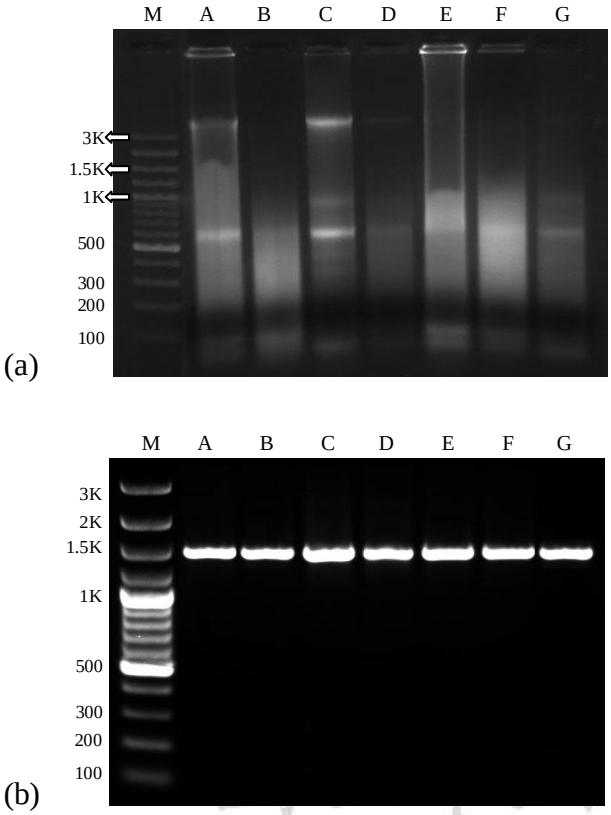


Figure 11. Electrophoretic analysis of (a) DNA extracts from lactic acid bacteria (b) PCR products from lactic acid bacteria. Lane M: MW marker Bio 100 bp ladder.

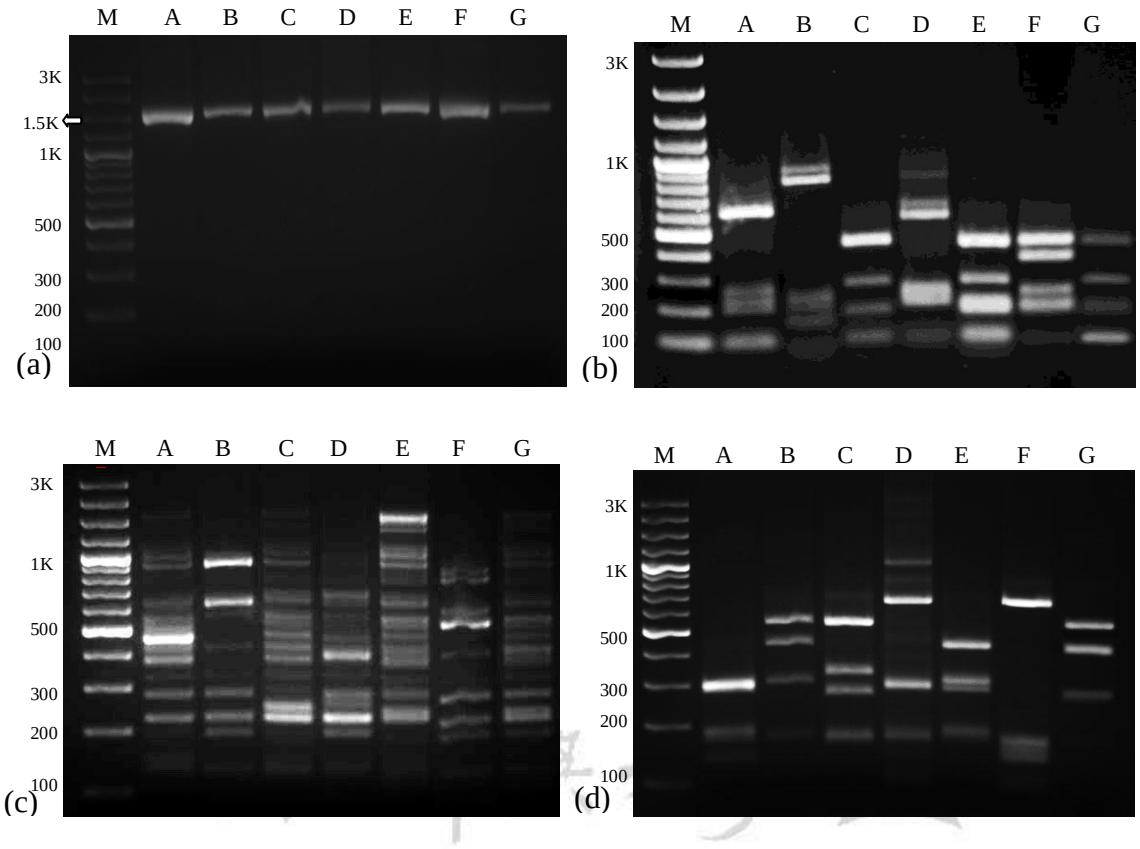


Figure 12. RFLP patterns obtained from digestion with four restriction enzymes (a) *Aci* I, (b) *Alu* I, (c) *Mse* I, and (d) *Dde* I. Lane M: MW ladder (100 bp); lane A: *L. plantarum*; lane B: *L. sakei*; lane C: *L. fermentum*; lane D: *L.brevis*; lane E: *P. pentosaceus*; lane F: *Ln. mesenteroides*; lane G: *Lc. lactis*.

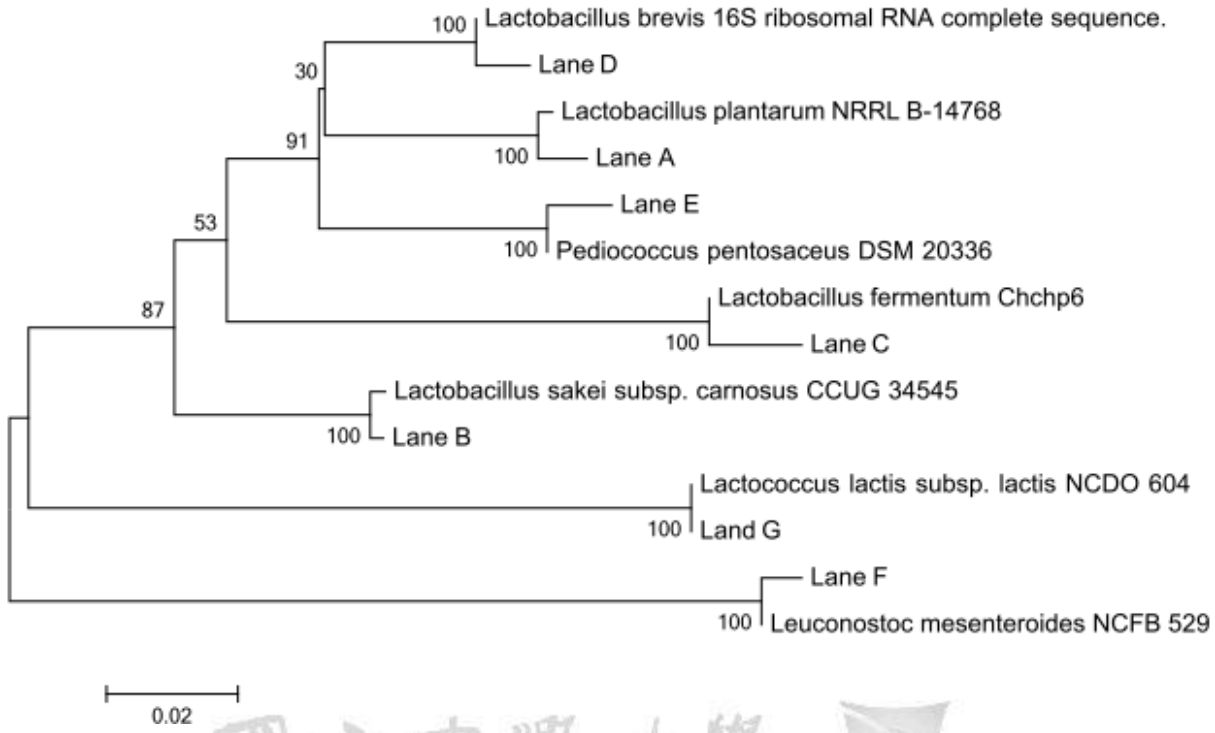


Figure 13. Phylogenetic relationship of LAB present in mum sausage based on maximum-likehood analysis of 16S rDNA sequences. The numbers indicate the confidence level (%) generated from 1000 bootstrap trees. The scale bar is in fixed nucleotide substitutions per sequence position.

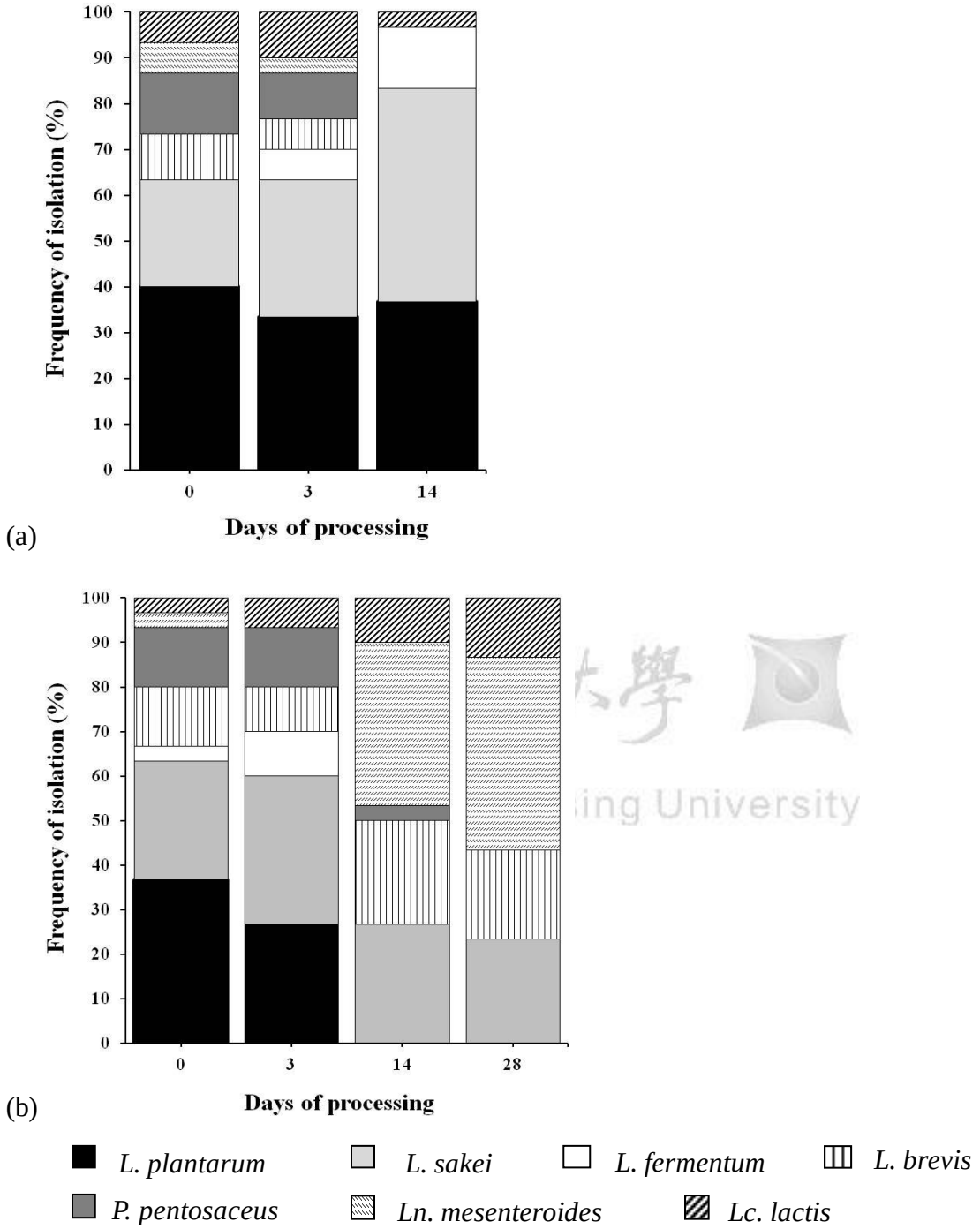


Figure 14. Lactic acid bacteria dynamics of (a) process I mum sausage (fermented and stored at ~30°C for 14 days) and (b) process II mum sausages (fermented at ~30°C for 3 days, vacuum-packaged, and stored at 4°C until day 28) during processing and storage.

Table 10 RFLP patterns and identification of lactic acid bacteria isolated from samples taken during the processing of mum sausages

RFLP pattern group [a]	Closest relative	% Identity [b]	Genbank accession no. [c]
A	*Lactobacillus plantarum*	98	NR042394.1
B	*Lactobacillus sakei*	98	NR025719.1
C	*Lactobacillus fermentum*	98	JQ446568.1
D	*Lactobacillus brevis*	98	NR044704.1
E	*Pediococcus pentosaceus*	97	NR042058.1
F	*Leuconostoc mesenteroides*	99	NR040817.1
G	*Lactococcus lactis*	99	NR040955.1

[a] Letters from A to G correspond to RFLP patterns in Fig. 12.

[b] Identical nucleotides percentage in the sequence obtained from PCR products and the sequence obtained found in NCBI.

[c] GenBank accession numbers of the sequences obtained from the representative isolates.

CHAPTER 5

The addition of starter cultures and nitrite to improve safety and quality of mum (Thai fermented sausages)

1. Abstract

Improvement of the conventional fermented sausage was investigated by introducing mixed starter cultures and curing agent. The objective of this study was to determine the effects of adding a mixed starter culture, which consisted of *Lactobacillus plantarum*, *Lactobacillus sakei* and *Lactococcus lactis*, and sodium nitrite ($NaNO_2$) on the physicochemical, microbial, textural, and sensory characteristics of mum (Thai fermented sausages). The results showed that the addition of selected mixed starter culture (*L. plantarum*, *L. sakei* and *Lc. lactis*) with $NaNO_2$ (125 ppm) had a great potential to improve quality and safety of mum sausages, which showed a significant decrease in pH values, inhibiting the growth of *Enterobacteriaceae*, and suppressing the accumulation of TBARS and VBN. Also, it showed an ability to reduce nitrite content and revealed a higher redness values. Moreover, sausages produced with mixed starter culture and nitrite had the highest scores in most sensory attributes especially in flavor, color, and overall acceptance.

Keywords: lactic acid bacteria, nitrite, Thai fermented sausage (mum), starter culture

2. Introduction

Mum is a Thai traditional dry fermented sausage, which made from beef, liver, and spleen with roasted rice powder, salt, and garlic. Raw material and ingredient are mixed well and stuffed into an animal intestine or bladder. Mum sausages are commonly fermented by indigenous microorganism for 2-3 days at room temperature (Thai Industrial Standards Institute, 2003). Many traditional fermented meat products in Thailand including mum sausages have been naturally fermented without the addition of starter cultures. This type of fermentation relies on the natural contamination by house flora, which occurs during animal slaughtering and sausage manufacturing (Ammor *et al*., 2005). Furthermore, this natural fermentation may contain some useful microorganism for the fermentation, flavor and texture development of the products, but it may also include several undesirable microorganisms including spoilage species and pathogens (Talon *et al*., 2007). The contamination of pathogenic microorganisms may cause food safety problems. *Staphylococcus aureus* and *Listeria monocytogenes* have been isolated from fermented pork

sausages (Paukatong and Kunawasen, 2001). These pathogens may grow under anaerobic condition and also produce some toxin (Gonzáles-Fandos *et al*., 1999). Additionally, the production of fermented sausage with natural condition always results in product with inconsistent qualities and even unsafe product (Wanangkarn *et al*., 2012).

The application of lactic acid bacteria (LAB) as starter culture can contribute to the production of dry fermented sausage with safety and standardization (Baka *et al*., 2011; Visessanguan *et al*., 2006). LAB improve product safety by inactivating spoilage and pathogenic microorganisms as a result of competitive growth and organic acid production (Ammor and Mayo, 2007), mainly lactic acid (Sriphochanart and Skolpap, 2011). Additionally, LAB can also produce some bacteriocins, such as nisin, which can use as natural food preservatives to prevent the growth of pathogens in fermented meat product (Noonpakdee *et al*., 2003). In case of sensory qualities, the addition of starter cultures can effectively improve the flavor, color, texture, and shelf-life of fermented meat product (Papamanoli *et al*., 2003; Lee *et al*., 2006). However, the LAB originating from fermented meats should be well adapted to the ecology of meat fermentation (Hugas and Monfort, 1997) and used as starter cultures. The most common LAB isolated from dry fermented sausages are *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Weissella*, and *Enterococcus* (Aymerich *et al*., 2006; Ammor *et al*., 2005). A strain of *Lactobacillus* is usually present as a dominant species (Ammor *et al*., 2005; Thiravattanamontri *et al*., 1998) and selectively used as starter cultures, especially *Lactobacillus plantarum* and *Lactobacillus sakei* (Hugas and Monfort, 1997; Baka *et al*., 2011; Hüfner and Hertel, 2008) and *Lactococcus lactis*, which produce nisin (Noonpakdee *et al*., 2003; Babji and Murthy, 2000).

Furthermore, for guarantee the final products to be safety and quality, manufacturers use not only a starter culture, but also some food additives, such as nitrite to develop color of fermented meat products, to inhibit spoilage and pathogenic bacteria, and to retard the rancidity of products (Maryuri *et al*., 2012). However, the addition of high level of nitrite may be a risk to human health due to reacting with amines to produce carcinogenic nitrosamines (Mirvish, 1995; Cammack *et al*., 1999). Due to the health concern, the maximum level of sodium nitrite limited by Ministry of Public Health of Thailand is 125 ppm of nitrite for meat products.

In our previous study, *L. plantarum* and *L. sakei* were identified as the major LAB species isolated from mum sausages, whereas the minority species such as *L. brevis, L. fermentum, P. pentosaceus, Ln. mesenteroides*, and *Lc. lactis* were also found (Wanangkarn *et al*., 2012). Therefore, the aim of this study was to evaluate the impact of the addition of mixed starter culture (consisting of *L. plantarum*, *L. sakei*, and *Lc. lactis*) and sodium nitrite on the physicochemical, microbial, textural, and sensory properties of mum sausages.

3. Materials and methods

3.1. Preparation of starter culture

Starter cultures were prepared by the method of Hu *et al*. (2007) with some modification. *Lactobacillus plantarum subsp. plantarum* ATCC 14917, *Lactobacillus sakei subsp. sakei* ATCC 15521, and *Lactococcus lactis subsp. lactis* ATCC 11454 were obtained from the Food Industry Research and Development Institute, Hsinchu, Taiwan. The LAB were subcultured in deMan Rogosa Sharpe (MRS) broth and purified by successively streaking MRS agar plates. The single colony was transferred in MRS broth and incubated at 30°C for 24 h. Cell pellets were harvested by a high speed refrigerated centrifuge (Himac centrifuge SCR20B, Hitachi, Japan) at 10,000 x g for 10 min at 4°C, and washed with saline water (0.85% NaCl), and then resuspended in the same saline solution. Finally, the number of bacterial cells in each suspension was adjusted to 7 log CFU/ml of saline solution (Sriphochanart and Skolpap, 2009; González-Fernández *et al*., 2006; Erkkila *et al*., 2001) by using a spectrophotometer at 600 nm (U3210, Hitachi, Japan).

3.2. Sausage preparation

Beef, spleen and liver were obtained from a local meat factory. After trimming of visible fat and connective tissue, meat and visceral organs were cut in pieces, vacuum-packed and kept frozen at -20°C before use. After thawing at 4°C overnight, raw materials, including beef (60% w/w), spleen (15% w/w), liver (15% w/w), were mixed thoroughly with non-meat ingredients, including ground garlic (4.2% w/w), roasted rice powder (42% w/w), and salt (1.6% w/w). The cell suspension of starter culture (10 ml/kg) was added to the meat mixture and mixed thoroughly. Five treatments of mum sausages were prepared as follows: prepared with different mixed starter cultures, namely, PS (*L. plantarum* subsp.

plantarum ATCC 14917 and *L. sakei* subsp. *sakei* ATCC 15521 [1:1]), PSL (*L. plantarum* subsp. *plantarum* ATCC 14917, *L. sakei* subsp. *sakei* ATCC 15521, and *Lc. lactis* subsp. *lactis* ATCC 11454 [1:1:1]), PS-N (*L. plantarum* subsp. *plantarum* ATCC 14917 and *L. sakei* subsp. *sakei* ATCC 15521 [1:1], and 125 ppm $NaNO_2$) and PSL-N (*L. plantarum* subsp. *plantarum* ATCC 14917, *L. sakei* subsp. *sakei* ATCC 15521, and *Lc. lactis* subsp. *lactis* ATCC 11454 [1:1:1], and 125 ppm $NaNO_2$), and CON (sterile saline water). After mixing, it was then stuffed into natural pig casings with a diameter of 3.0 cm and a length of 15 cm (approximately 250-300 g each). Before operation, mixer (Kilia Fleischinenfabric, Kiel, Germany) and stuffer (Dick D-73779, Germany) were first cleaned with hot water, dried with paper towel and disinfected with 75% ethanol. Sausages were hung vertically on stainless steel hangers at 30±3°C and 65±2% relative humidity until day 3 for the fermentation purpose, then vacuum-packaged (HAS02G, Europack, Holland) in a vacuum bag (laminate of Nylon/LLDPE, Chun I Gravure Co. Ltd., Taichung, Taiwan), and stored at 4°C until day 28. Sausages were sampled on day 0 (after stuffing), day 3 (end-fermentation), day 14 (storage process), and day 28 (storage process) for analyses.

3.3. Physicochemical analysis

3.3.1. Proximate composition

Samples were ground with a grinder (Type 780A, Krups, Ireland). Proximate compositions of samples, including moisture, crude protein, crude fat, and ash contents, were measured according to the AOAC (1990) methods. Crude protein was measured by the Kjeldahl method, using a digester (Model 2006, Foss tecator, Sweden) and a distillation unit (Model 2100, Foss tecator, Sweden). Crude fat was measured using a fat extractor (Sotec System HT 1043 Extraction Unit, Tecator Co., Sweden) with ethyl ether as a solvent and extracted for 16 h.

3.3.2. Water activity, pH and titratable acidity measurement

Water activity (a_w) was measured using a water activity analyzer (Aqualab-CX2, Decagon Devices Inc., USA). Ten-gram samples were blended with 90 ml distilled water in a polyethylene bag for 2 min using a laboratory blender, and then the pH value of mixture was measured using a pH meter (Weilheim, Germany). Titratable acidity was determined as percentage of lactic acid by titrating with 0.1 N NaOH, using phenolphthalein as an

indicator (AOAC, 1990).

3.3.3. Determination of Thiobarbituric acid reactive substances (TBARS), volatile basic nitrogen (VBN) and nitrite residue.

TBARS values of the samples were determined according to the method of Faustman *et al.* (1992). TBARS value was expressed as mg malonaldehyde/kg meat. Volatile basic nitrogen was determined according to CNS (1982) using a Conway micropipette diffusion method. Nitrite residue was measured according to the methods of Liu *et al.* (2010) and residual nitrite concentration (ppm) was measured by using a Spectrophotometer (U3210, Hitachi, Japan) at 540 nm wave-length.

3.3.4. Instrumental color measurement and texture profile analysis (TPA)

Color parameters of samples were measured with a handy colorimeter (NR-300, Nippon Denshoku, Japan). The instrument was first standardized with a calibration plate with 'Y' = 86.53, 'X' = 82.45, and 'Z' = 91.28. Six measurements of surface reflectance which expressed as L* (lightness), a* (redness), and b* (yellowness) values were performed on the slices of sausages with a thickness of approximately 3 cm and averaged. In texture profile analysis (TPA), a texture analyzer (Compact-100, Sun Rheo Meter, Japan) was applied to determine texture parameters, including hardness, springiness, cohesiveness, chewiness, gumminess, and gel strength of samples. This procedure involved cutting five slices of sausage samples (approximately 2.0 cm in height and 2.0 cm in diameter) after discarding the external layer. Samples were allowed to equilibrate to room temperature and then axially compressed into two consecutive cycles of 50% of the original height, with a 30-mm diameter probe. Force-time deformation curves were obtained with a 10 kg load cell which was applied at a cross-head speed of 1 mm/s (Bourne, 1978).

3.4. Microbiological analysis

Microbial qualities were determined accordingly (APHA, 2001). At a specified sampling time, 25-gram sample was aseptically placed in a sterile bag, which contained 225 ml of 0.85% NaCl solution, and homogenized with a stomacher (Stomacher blender, Model 400, Seward) for 2 min. Serial dilutions were then made. Plate count agar (Merck, Dram Stadt, Germany) and violet red bile agar (Merck, Dram Stadt, Germany) were used for enumeration of total plate counts and *Enterobacteriaceae* counts, respectively, using the

pour plate method. Total microflora and *Enterobacteriaceae* were incubated at 35°C for 24 h. For lactic bacteria counts, dilutions were poured into MRS agar (Merck, Dram Stadt, Germany), placed in an anaerobic jar (BBL GasPak System, USA) and incubated at 37°C for 48 h. The microbial counts were expressed as $\log_{10}$ colony forming units (CFU) per gram of sample.

3.5. Sensory evaluation

At the end of fermentation and storage process (i.e. day 3, day 14 and day 28, respectively), sausages were first cooked on a grill, until the internal temperature of the sausages reached and was held at 75°C for 8 min, cooled to room temperature (approximately 25°C), sliced (approximately 1.0 cm thickness), and then served to a sensory panel which consisted of 10 panelists. Sensory attributes, including color, flavor, texture, sourness, saltiness, and overall acceptability, were evaluated using a 1 to 7-point hedonic scale test, with 1 and 7 representing extremely dislike and extremely like, respectively, for the attributes.

3.6. Statistical analysis

Analysis of variance (ANOVA) was performed to analyze the effect of treatment (CON, PS, PSL, PS-N, and PSL-N) and time of storage. Mean comparisons were run by Duncan's multiple range test. Data analyses were performed using Statistical Analysis System's Procedures (Version 9.1, SAS Institute Inc., Cary, NC) with a 5% level of significance.

4. Results and discussion

4.1. Physicochemical analysis

Changes in proximate composition and water activity of mum sausages were presented in Table 11. Moisture contents of all samples decreased significantly from 69-70% at day 0 (after stuffing) to 56-59% at day 3 and slightly decreased to 51-57% at day 14 and 28. In this study, sample that inoculated with starter cultures had significantly lower moisture content than controls when fermented and stored up to 28 days. Conversely, there was significant increase ($P < 0.05$) in crude protein, crude fat, and ash contents, while moisture decreased during storage for all treatments.

Change pattern in moisture content of all samples, which consisted of a rapid decrease during fermentation and followed by a slow decrease during storage, was also observed in vacuum-packaged and naturally fermented mum sausages (Wanangkarn *et al*., 2012). Similar results were also reported by Dalmış and Soyer (2008) and González-Fernández *et al*. (2006), who found higher moisture content in the uninoculated than the inoculated sausages during storage. However, Kato *et al*. (1985) indicated that pH and water holding capacity of fermented sausages decreased with increasing concentration of starter cultures and incubation time. Huff-Lonergan and Lonergan (2005) reported that the development of low water holding capacity and unacceptably high purge loss was probably related to the accelerated pH decline. Riebroy *et al*. (2008) explained that the coagulation or aggregation of proteins due to acid affected water-holding capacity in meat.

The results in Table 12 showed that the averaged water activity (a_w) of the sausages decreased significantly from 0.95 at day 0 to 0.91–0.92 at day 28. Significant changes in moisture and a_w of samples were observed after fermentation, and it was probably due to the high temperature (30°C) and low relative humidity (65% RH). In this study, it was found that mixed starter culture sausages had lower a_w and pH value than control ($P < 0.05$). Lee *et al*. (1981) reported that a correlation (R^2=0.98) was observed between water activity and moisture content in pre-cooked bacon. Pérez-Alvarez *et al*. (1999) explained that the change in moisture content during drying process was probably due to the evaporation of free surface water and the free internal water diffuses towards the periphery. In this study, the small decrease of a_w in mum sausages was possible due to the protection of vacuum-packaging during storage and it agreed with previous study (Wanangkarn *et al*., 2012). The low a_w might extend the lag phase of the growth of undesirable microorganisms, reduced the logarithmic phase and consequently lowered the microorganism level in the stationary phase. Thus, a_w must be reduced as quickly as possible to inhibit or to delay the growth of spoilage microorganisms in the product (Kalalou *et al*., 2004).

The change of pH values in samples was given in Table 12. The initial mean pH value of sausage batter was 5.79-5.86, and no differences ($P > 0.05$) were found among the treatments. The pH was decreased significantly ($P < 0.05$) on day 3 in all samples, and sausages inoculated with starters exhibited significantly lower pH than control. At the end

of fermentation, pH of samples, which inoculated with starter culture and/or nitrite including PS, PSL, PS-N, and PSL-N treatments, were 4.18, 4.23, 4.25, and 4.28, respectively, while the control sample was 4.48. Change of pH value in fermented sausages also paralleled a titratable acidity. With a rapid decrease in pH values during fermentation, titratable acidity increased significantly ($P < 0.05$) on day 3 and then remained constantly until day 28. In this study, the titratable acidity in sausages fermented with starter cultures was higher than that of control samples during the whole storage period. Pérez-Alvarez *et al.* (1999) reported that a statistically significant correlation (R^2=0.887) was observed between pH value and lactic acid content in Spanish dry cured sausages. This reduction in pH was due to formation of lactic acid (Antara *et al*., 2004) by LAB that fermented carbohydrate as an energy source (Baka *et al*., 2011; Visessanguan *et al*., 2006).

However, the pH value should be rapidly decreased during fermentation to inhibit undesirable microorganisms (Ferreira *et al*., 2006). It was recommended that uncooked sausages normally eaten with pH lower than 4.5 were safe for consumption (Paukatong and Kunawasen, 2001), and Juneja and Novak (2003) indicated that 90% of the population of *Escherichia coli* O157:H7 in ground beef were inactivated at pH 4.5. In this study, after day 28, the pH value was slightly increased in all treatments but lower than 4.5 in the starter culture ones (4.23-4.30) except only control batch (4.53). This final pH increase in meat and meat products could be due to the proteolytic activity of microorganisms generated peptides, amino acids, and amines (Pérez-Alvarez *et al*., 1999; Roig-Sagués *et al*., 1999), which had a buffering effect on the organic acids (Cocolin *et al*., 2001).

TBARS value, which is a indicator of lipid oxidation, was shown in Fig. 13. The TBARS value of all samples increased throughout storage time as expected. The rapid increase in TBARS value occurred in control sausages from 1.5 mg MDA/Kg at day 0 (after stuffing) up to 4.54 mg MDA/Kg at day 3 (end-fermentation), while in sausages inoculated with starter culture increased from 0.99-1.12 mg MDA/Kg at day 0 (after stuffing) up to 2.15-2.69 mg MDA/Kg at day 3, and followed by a slow increase to 2.68-3.46 and 3.99-4.70 mg MDA/Kg at day 14 and 28, respectively. In control sausages, TBARS value increased throughout storage time and exhibited the highest final value as 6.10 mg MDA/Kg. In this study, the TBARS value of final products with addition of

sodium nitrite (PS-N and PSL-N) were lower than those of control and only starter culture inoculate samples.

This increase of TBARS value in Thai fermented sausage has also been reported by other researchers. Noonpakdee *et al*. (2003) reported that TBARS value increased from 2.82 mg MDA/Kg up to 3.22 at day 3 of fermentation in sausage inoculated with *L. plantarum*. The authors explained that hydrogen peroxide produced from LAB was the main factor in lipid oxidation of the fermented meat, and should use a starter culture with catalase production. According to the report of Bozkurt and Erkmen (2004), the variation of TBARS value in sample might be due to the addition of antioxidants (nitrite, ascorbic acid, and tocopherols) and different manufacturing techniques (starter culture type and poor quality of the raw material). The results of this study showed that the addition of starter cultures and nitrite could retard the lipid oxidation and agreed with other studies. When evaluating the TBARS value of samples, Sawitzki *et al*. (2008) report that Milano-type salami inoculated with *L. plantarum* strain AJ2 showed lower TBARS values when compared to the control throughout the storage time.

Macdonald *et al*. (1980) reported that the concentration of nitrite should be greater than 25 ppm for substantially reduced rates of oxidation. According to Honikel (2008), nitrite could limit lipid oxidation by reacting with water to form HNO_2 and HNO_3, and sequestering oxygen. Thus, the development of rancidity or a warmed over flavor were retarded.

VBN value of all sausages samples increased during processing. Samples with addition of starter cultures had significantly lower VBN values than control samples. Additionally, inoculated samples containing sodium nitrite (PS-N and PSL-N) had significantly ($P < 0.05$) lower VBN values than sausage inoculated only starter culture (PS and PSL). This result agreed with other studies (Hu *et al*., 2007; Yin and Jiang, 2001). Yin *et al*. (2002) indicated that the combination of *L. plantarum* CCRC10069, and *Lc. lactis subsp. lactis* CCRC12315 could inhibit the accumulation of VBN due to their lactic acid production and antimicrobial bacteriocins, which could neutralize the volatile basic nitrogen. Additionally, the VBN values not only indicate freshness of meat and meat products, but also are related to some biogenic amines contents in meats (Min *et al*., 2007).

The residual nitrite contents of PS-N and PSL-N decreased rapidly from 113.6 and 111.9 ppm at day 0 to 48.6 and 36.0 ppm at day 3, and eventually 22.4 and 9.3 ppm at day 28, respectively. This reduction was more than 50% of an initial content, and still significantly decreased until day 28. Additionally, PSL-N showed a lower nitrite content than PS-N in final products. Esmaeilzadeh *et al*. (2012) evaluated the ability of pure starter culture to deplete nitrite during fermentation. The authors reported that *L. plantarum* and *L. fermentum* showed higher ability to reduce nitrite contents. Additionally, a statistically significant correlation (R^2=0.91) was observed between pH value and nitrite content in fermented sausages (Baka *et al*., 2011). However, Honikel (2008) indicated that the regulations of the European Parlia-ment and Council Directive 95/2/EC defined that the nitrite residual should be ≤ 50 mg nitrite/kg in non-heat treated meat products and ≤ 100 mg nitrite/kg in all other meat products except minor.

4.2. Instrumental color evaluation

Changes in L* (lightness), b* (yellowness), and a* (redness) values during the fermentation and storage of fermented sausages were shown in Fig. 14. L* and b* value of all samples were increased significantly during 3 days of fermentation and slightly increased throughout the storage time. Similar change patterns of L* and b* value were also observed by Wanangkarn *et al*. (2012) and Liaros *et al*. (2009), as sausages were vacuum-packaged after fermentation. According to the conclusion of Pérez-Alvarez *et al*. (1997), the increase in L* observed during fermentation could be attributed to the effect of lactic acid and pH, which caused exudation in the meat. Additionally, Visessanguan *et al*. (2005) also explained that acidification of meat proteins could lead to shrinkage of the myofilament lattice which increasing the light reflected from the meat.

The a* values (redness) was significantly decreased, especially ($P < 0.05$) during fermentation (day 3) and up to the end of the storage time (day 28) in the control and mix starter culture batches (PS and PSL) without nitrite addition. In this study, a* value of the sample with nitrite (PS-N and PSL-N) was significantly increased and showed the highest value during the fermentation period (day 3), when compared with sausages inoculated starter culture, and then slightly decreased throughout the storage time. However, there was no significant different between PS-N and PSL-N batches. The decrease in a* values during

the storage of fermented sausages was probably due to the effect of hydrogen peroxide (H_2O_2) produced by LAB, which contributed to oxidative discoloration of sausages. On the contrary, Baka *et al*. (2011) indicated that some strains of *L. sakei* had the ability to improve redness by producing heme dependent catalase. According to Møller *et al*. (2003), sausages inoculated with some Lactobacillus had ability to generate NO that could form the basis for production of cured meat products without using nitrate/nitrite. Kawahara *et al*. (2006) also found that acceptable shelf stability and color in cured meat products might be attained by the use of *L. sakei* as a starter culture. Therefore, NO producing bacterial strains could become an alternative to the usage of nitrate and nitrite in fermented meat product. Color formation is one of the important quality attribute of cure meat product which effects by nitrite, which converts to the nitrosylmyoglobin (MbFe(II)NO) and contributes pink cured meat color (Honikel, 2008; Bozkurt and Erkmen, 2004)

4.3. Microbial qualities

Microbial changes in samples during storage were shown in Fig. 15. The total plate counts (TPC) of control batch increased significantly from 5.7 log CFU/g at day 0 up to 8.5 log CFU/g at day 3, and then decreased to 8.0 log CFU/g at day 28. In the batch inoculated with starters, the TPC had an initial level of 7.1-7.2 log CFU/g. The TPC increased to 10.5-10.8 log CFU/g during the fermentation (day 3) and then slightly decreased to 9.6-10.2 log CFU/g at day 14.

Similarly, the lactic bacteria (LAB) counts in control samples increased significantly from 5.6 log CFU/g at day 0 up to 7.9 log CFU/g at day 3, and then slightly decreased to 7.6 and 7.4 log CFU/g at day 14 and day 28, respectively. In the batch inoculated with starters, the LAB counts had an initial level of 7.2 log CFU/g and rapidly increased to 10.2-10.5 log CFU/g at day 3, and then decreased to 9.6-10.2 and 9.3-9.9 log CFU/g at day 14 and 28, respectively. Additionally, the LAB counts in starter samples were higher than that of the control samples throughout processing.

A similar microbial change pattern was observed between the total microorganisms and lactic acid bacteria during processing, and was in agreement with Sripochanart and Skolpap (2009), who investigated the use of various starter cultures in Thai fermented sausage and reported that the total microorganisms was higher than LAB counts due to the

population of other microorganism. However, lactic acid bacteria became predominant after fermentation stage of Thai fermented sausages and other types of fermented sausages (Antara *et al*., 2004; Roig-Sagués *et al*., 1999; Rubio *et al*., 2008). The use of LAB starter cultures that originate from mum sausage might be well adapted to the ecology of mum fermentation by present faster growth rates during sausage fermentation and ripening (Baka *et al*., 2011; Rubio *et al*., 2008). González-Fernández *et al*. (2006) and Baka *et al*. (2011) indicated that the rapid increase in LAB counts occurred in the sausages inoculated with *L. sakei* and *L. plantarum*. However, LAB counts might be decreased due to water activity decrease or the other unfavorable conditions (Kalalou *et al*., 2004). In this study, the initial *Enterobacteriaceae* counts in sausage ranged from 4.5-4.6 log CFU/g at day 0 and decreased to 2.2-3.8 log CFU/g at day 3. No *Enterobacteriaceae* was detected in sausages with starter cultures at day 14, whereas in the control batch remained to present 2.8 log CFU/g and disappeared at day 28.

The organic acids were produced by LAB could reduce pH, and action of these acids is related to the ability of the undissociated acid molecules to penetrate through the bacterial plasma membrane. In the cytoplasm, the acid dissociates to release protons and conjugate bases under high pH. This disrupts the membrane proton-motive force, thus disabling the energy-yielding and transport process dependent upon it (Savard *et al*., 2002). Moreover, Castáno *et al*. (2002) reported that a significant correlation of the *Enterobacteriaceae* count with a_w, salt/moisture, and pH value were 0.94, -0.94 and 0.69, respectively. Some bacteriocins produced by LAB inhibited not only closely related species, but also effective against food-borne pathogens, such as *Listeria monocytogenes*, *Clostridium botulinum*, and *Staphylococcus aureus* (Lewus *et al.*, 1991), and spoilage microorganisms, such as *Bacillus sp*. and *Enterococcus faecalis* (Delves-Broughton, 1990). *Lc. lactis* strain WNC 20 is able to produce the bactericocin nisin-Z originally isolated from Nham (Noonpakdee *et al*., 2003). *L. sakei* or *L. plantarum* U201 can produce some bacteriocin and inhibits the growth of *Enterobacteriaceae* below 2.0 log CFU/g (González and Díez, 2002; Talon *et al*., 2008; Antara *et al*., 2004).

According to Roig-Sagués *et al*. (1999), various factors, such as a low initial pH value and water activity, a high concentration of fermentable carbohydrates, high numbers

of *lactobacilli* in the fresh sausage mixture, the presence of 50-100 ppm nitrite, and low ripening temperatures, could suppress the growth of *Enterobacteriaceae* (González and Diez, 2002). Woods *et al*. (1981) explained that nitrite could be reduced to nitric oxide, which were toxic to a variety of microorganisms.

4.4. Texture and sensory characteristics

The results of texture profile analysis were shown in Table 15. The results showed that the hardness of all samples rapidly increased from day 0 to day 3 and slightly increased to day 28. However, the highest value of hardness was observed in control batches, whereas the lowest was observed in PS samples. Similar change patterns were also observed in chewiness, adhesiveness, and gel strength. Springiness and cohesiveness of all samples decreased significantly at day 3, and these two characteristics remained stable to day 28. Casquete *et al*. (2011) explained that the reduction of hardness in sausage with starter cultures resulted from proteolytic activity by lactic acid bacteria. Martín *et al*. (2007) indicated that LAB could produce proteases and peptidases, and contributed significantly to the breakdown of myofibrillar proteins. These results supported that starter cultures decreased the hardness of mum samples due to protein hydrolysis.

Fig. 16 illustrated the sensory evaluation results. All treatments inoculated with starter culture (PS, PSL, PS-N, and PSL-N) had higher scores for all sensory attributes than control samples at all storage time (ie. day 3, 14 and 28). However, the treatments with starter cultures and nitrite (PS-N and PSL-N) had higher scores for color, flavor and overall acceptance than the treatment without nitrite (PS and PSL). No significant differences were found between PS-N and PSL-N treatments. In this study, flavor, saltiness and overall acceptance tended to decrease as storage time increased.

According to the report of Kameník *et al*. (2012), the reduction of sensory scores might be due to the microorganisms enzymes that were functional with muscle endogenous enzymes to catalyze biochemical reactions, resulting in a negative effect on taste and aroma at later stages of storage. *L. sakei* and *Lc. lactis* could improve flavor of dry fermented sausage by producing exopeptidases, which hydrolyzed protein and generated free amino acids and peptides to improve flavor (Demeyer *et al*., 2000; Herranz *et al*., 2004). Honikel (2008) indicated that nitrite improved red color, retarded microbial growth and gave a

pleasant favor. This might explain why the PSL-N treatment showed the better sensory qualities than other treatments in this study.

Table 11 Changes in proximate composition of mum sausages with/without starter cultures and nitrite during storage

Treatment	Storage time (day)			
	0	3	14	28
Moisture (%)				
Con	69.91±0.36 a,A	58.22±0.15 b,A	57.12±0.26 c,A	54.99±0.43 d,A
PS	69.81±0.66 a,A	56.03±0.24 b,B	53.70±0.43 c,B	51.76±0.58 d,B
PSL	70.07±0.64 a,A	56.06±0.96 b,B	53.79±0.50 c,B	51.87±0.46 c,B
PS-N	69.79±0.36 a,A	56.38±0.14 b,B	54.13±0.48 c,B	52.44±0.22 d,B
PSL-N	69.40±0.41 a,A	56.18±0.37 b,B	53.89±0.24 c,B	52.50±0.49 d,B
Protein (%)				
Con	19.22±0.43 c,A	29.08±0.27 b,A	29.81±0.17 b,C	31.76±0.35 a,B
PS	19.68±0.18 d,A	30.02±0.37 c,A	31.54±0.17 b,A	33.65±0.17 a,A
PSL	18.86±0.29 d,A	29.72±0.25 c,A	31.39±0.13 b,B	33.37±0.18 a,A
PS-N	19.33±0.40 d,A	29.44±0.21 c,A	30.56±0.37 b,BC	32.90±0.25 a,A
PSL-N	18.87±0.30 d,A	29.60±0.33 c,A	30.59±0.32 b,BC	33.19±0.16 a,A
Fat (%)				
Con	4.41±0.02 c,AB	5.95±0.05 b,A	6.09±0.02 a,B	6.17±0.05 a,B
PS	4.35±0.03 c,AB	6.18±0.05 b,A	6.27±0.08 ab,A	6.37±0.04 a,AB
PSL	4.48±0.05 c,A	6.13±0.10 b,A	6.22±0.01 ab,AB	6.39±0.06 a,A
PS-N	4.41±0.07 b,AB	6.09±0.10 a,A	6.14±0.03 a,AB	6.25±0.06 a,AB
PSL-N	4.23±0.08 b,B	6.11±0.10 a,A	6.20±0.06 a,AB	6.28±0.08 a,AB
Ash (%)				
Con	3.09±0.01 d,A	3.83±0.02 c,C	3.93±0.02 b,C	4.10±0.02 a,C
PS	3.08±0.02 d,A	4.07±0.01 c,A	4.17±0.01 b,A	4.25±0.01 a,A
PSL	3.08±0.02 d,A	4.05±0.02 c,A	4.15±0.01 b,A	4.24±0.01 a,A
PS-N	3.10±0.01 d,A	3.98±0.03 c,B	4.11±0.01 b,B	4.21±0.01 a,AB
PSL-N	3.11±0.01 d,A	4.02±0.01 c,AB	4.14±0.01 b,AB	4.19±0.01 a,B

[a–d] Means within the same row with different superscripts are significantly different ($P < 0.05$).

[A–C] Means within the same column with different superscripts are significantly different ($P < 0.05$).

Con=Control (no starter culture or nitrite); PS= starter culture (*L. plantarum* and *L. sakei*) added; PSL= starter culture *(L. plantarum*, *L. sakei* and *Lc. lactis*) added ; PS-N=starter culture (*L. plantarum* and *L. sakei*) and 125 ppm nitrite added ; PSL-N= starter culture (*L. plantarum*, *L. sakei* and *Lc. lactis*) and 125 ppm nitrite added.

Table 12 Changes in water activity, pH value and titratable acidity of mum sausages with/without starter cultures and nitrite during storage

Treatment	Storage time (day)			
	0	3	14	28
Water activity				
Con	0.957±0.001 ^{a,A}	0.941±0.001 ^{b,A}	0.937±0.001 ^{c,A}	0.922±0.001 ^{d,A}
PS	0.959±0.002 ^{a,A}	0.934±0.002 ^{b,B}	0.928±0.003 ^{b,B}	0.910±0.001 ^{c,B}
PSL	0.957±0.002 ^{a,A}	0.935±0.002 ^{b,B}	0.930±0.002 ^{b,AB}	0.913±0.001 ^{c,B}
PS-N	0.955±0.002 ^{a,A}	0.936±0.001 ^{b,B}	0.929±0.003 ^{c,B}	0.911±0.001 ^{d,B}
PSL-N	0.957±0.001 ^{a,A}	0.938±0.001 ^{b,AB}	0.931±0.002 ^{c,AB}	0.912±0.001 ^{d,B}
pH value				
Con	5.79±0.02 ^{a,A}	4.48±0.02 ^{b,A}	4.49±0.03 ^{b,A}	4.53±0.01 ^{b,A}
PS	5.80±0.05 ^{a,A}	4.18±0.03 ^{b,C}	4.15±0.03 ^{b,C}	4.23±0.02 ^{b,B}
PSL	5.83±0.04 ^{a,A}	4.23±0.02 ^{b,BC}	4.20±0.02 ^{b,BC}	4.26±0.03 ^{b,B}
PS-N	5.86±0.01 ^{a,A}	4.25±0.01 ^{bc,BC}	4.26±0.02 ^{c,B}	4.30±0.01 ^{b,B}
PSL-N	5.81±0.03 ^{a,A}	4.28±0.01 ^{b,B}	4.27±0.00 ^{b,B}	4.30±0.05 ^{b,B}
Titratable acidity (%)				
Con	0.61±0.04 ^{c,A}	2.33±0.02 ^{a,D}	2.11±0.01 ^{b,E}	2.13±0.02 ^{b,D}
PS	0.64±0.03 ^{c,A}	2.96±0.03 ^{a,A}	2.93±0.01 ^{a,A}	2.83±0.04 ^{b,A}
PSL	0.62±0.03 ^{c,A}	2.85±0.02 ^{a,B}	2.83±0.01 ^{a,B}	2.75±0.02 ^{b,B}
PS-N	0.63±0.03 ^{c,A}	2.72±0.01 ^{b,C}	2.78±0.01 ^{a,C}	2.70±0.01 ^{b,BC}
PSL-N	0.62±0.04 ^{b,A}	2.69±0.02 ^{a,C}	2.73±0.02 ^{a,D}	2.64±0.03 ^{a,C}

$^{a–d}$ Means within the same row with different superscripts are significantly different ($P < 0.05$).

$^{A–D}$ Means within the same column with different superscripts are significantly different ($P < 0.05$).

Con, PS, PSL, PS-N, and PSL-N: abbreviations are the same as Table 11.

Table 13 Changes in TBA, VBN and nitrite content of mum sausages with/without starter cultures and nitrite during storage

Treatment	Storage time (day)			
	0	3	14	28
TBA (mg malonaldehyde/Kg)				
Con	1.05±0.06 ^{d,A}	4.54±0.12 ^{c,A}	5.39±0.08 ^{b,A}	6.10±0.08 ^{a,A}
PS	0.99±0.03 ^{d,A}	2.69±0.10 ^{c,B}	3.46±0.10 $^{b.B}$	4.70±0.14 ^{a,B}
PSL	1.02±0.03 ^{d,A}	2.36±0.05 ^{c,C}	3.37±0.10 $^{b.B}$	4.51±0.02 ^{a,BC}
PS-N	1.12±0.02 ^{d,A}	2.25±0.06 ^{c,C}	3.25±0.02 $^{b.B}$	4.32±0.02 ^{a,C}
PSL-N	1.09±0.02 ^{d,A}	2.15±0.12 ^{c,C}	2.68±0.04 $^{b.C}$	3.99±0.03 ^{a,D}
VBN (mg/ 100 g)				
Con	17.60±0.35 ^{d,A}	49.38±0.63 ^{c,A}	52.99±0.69 ^{b,A}	56.39±0.69 ^{a,A}
PS	17.55±0.04 ^{d,A}	46.57±0.47 ^{c,B}	50.14±0.47 ^{b,B}	53.79±0.47 ^{a,B}
PSL	17.72±0.58 ^{d,A}	44.66±0.32 ^{c,C}	47.82±0.32 ^{b,C}	51.17±0.32 ^{a,C}
PS-N	17.58±0.12 ^{d,A}	43.33±0.18 ^{c,C}	46.72±0.18 ^{b,CD}	50.68±0.18 ^{a,C}
PSL-N	18.09±0.45 ^{d,A}	41.82±0.57 ^{c,D}	45.44±0.57 ^{b,D}	48.82±0.57 ^{a,D}
Nitrite content (ppm)				
PS-N	113.61±0.90 ^{a,A}	48.60±1.06 ^{b,A}	31.59±0.88 ^{c,A}	22.35±0.57 ^{d,A}
PSL-N	111.94±0.66 ^{a,A}	36.03±0.81 ^{b,B}	18.94±0.48 ^{c,B}	9.30±0.60 ^{d,B}

$^{a–d}$ Means within the same row with different superscripts are significantly different ($P < 0.05$).

$^{A–D}$ Means within the same column with different superscripts are significantly different ($P < 0.05$).

Con, PS, PSL, PS-N, and PSL-N: abbreviations are the same as Table 11.

Table 14 Changes in color parameters of mum sausages with/without starter cultures and nitrite during storage

Treatment	Storage time (day)			
	0	3	14	28
L* value				
Con	36.63±0.53 ^{b,A}	46.66±0.59 ^{a,A}	46.87±0.50 ^{a,A}	47.12±0.52 ^{a,A}
PS	36.20±0.53 ^{c,A}	41.83±0.19 ^{b,C}	42.66±0.29 ^{ab,C}	43.88±0.74 ^{a,B}
PSL	37.57±0.53 ^{c,A}	42.12±0.09 ^{b,C}	43.41±0.29 ^{ab,C}	44.65±0.76 ^{a,AB}
PS-N	37.60±0.53 ^{b,A}	43.95±0.24 ^{a,B}	45.57±0.81 ^{a,AB}	45.89±1.15 ^{a,AB}
PSL-N	37.61±0.53 ^{b,A}	44.62±0.96 ^{a,B}	45.16±0.24 ^{a,B}	45.85±0.79 ^{a,AB}
a* value				
Con	22.96±0.26 ^{a,A}	18.13±0.15 ^{b,C}	16.14±0.28 ^{c,B}	15.96±0.15 ^{c,B}
PS	23.34±0.27 ^{a,A}	19.59±0.52 ^{b,B}	17.24±0.79 ^{c,B}	16.54±0.32 ^{c,B}
PSL	22.04±0.27 ^{a,B}	18.88±0.16 ^{b,BC}	17.64±0.32 ^{c,B}	16.57±0.41 ^{d,B}
PS-N	21.66±0.27 ^{a,B}	33.45±0.38 ^{b,A}	31.85±0.40 ^{c,A}	30.29±0.28 ^{d,A}
PSL-N	21.95±0.26 ^{a,B}	33.85±0.62 ^{b,A}	31.3±0.340 ^{c,A}	29.42±0.58 ^{d,A}
b* value				
Con	12.67±0.55 ^{b,A}	14.03±0.51 ^{ab,A}	14.38±0.53 ^{a,AB}	14.75±0.41 ^{a,A}
PS	11.78±0.54 ^{c,A}	12.80±0.37 ^{bc,AB}	13.69±0.43 ^{ab,BC}	14.44±0.39 ^{a,A}
PSL	12.70±0.55 ^{b,A}	14.04±0.59 ^{ab,A}	15.05±0.04 ^{a,A}	15.52±0.37 ^{a,A}
PS-N	11.32±0.54 ^{b,A}	11.48±0.59 ^{b,B}	12.54±0.41 ^{b,C}	14.60±0.30 ^{a,A}
PSL-N	11.29±0.23 ^{c,A}	13.55±0.42 ^{b,A}	14.44±0.28 ^{b,AB}	15.88±0.53 ^{a,A}

$^{a–d}$ Means within the same row with different superscripts are significantly different ($P < 0.05$).

$^{A–C}$ Means within the same column with different superscripts are significantly different ($P < 0.05$).

Con, PS, PSL, PS-N, and PSL-N: abbreviations are the same as Table 11.

Table 15 Changes in texture profiles of mum sausages with/without starter cultures and nitrite during storage

Treatment	Storage time (day)			
	0	3	14	28
Hardness (N)				
Con	5.50±0.41 ^{b,A}	37.56±1.36 ^{a,A}	38.15±0.24 ^{a,A}	39.01±0.24 ^{a,A}
PS	5.75±0.60 ^{c,A}	31.41±0.71 ^{b,C}	33.47±0.33 ^{a,C}	35.23±0.66 ^{a,C}
PSL	6.00±0.73 ^{c,A}	36.08±0.34 ^{ab,AB}	35.28±0.37 ^{b,B}	37.01±0.39 ^{a,B}
PS-N	5.77±0.56 ^{c,A}	35.79±0.45 ^{b,AB}	36.46±0.50 ^{b,AB}	38.27±0.45 ^{a,AB}
PSL-N	5.74±0.48 ^{c,A}	34.97±0.21 ^{b,B}	36.21±0.96 ^{ab,B}	37.13±0.43 ^{a,B}
Springiness (mm)				
Con	0.69±0.01 ^{a,A}	0.48±0.02 ^{b,A}	0.46±0.02 ^{b,A}	0.49±0.02 ^{b,A}
PS	0.66±0.01 ^{a,A}	0.41±0.02 ^{b,A}	0.38±0.01 $^{b.B}$	0.39±0.01 ^{b,B}
PSL	0.71±0.04 ^{a,A}	0.43±0.02 ^{b,A}	0.40±0.02 $^{b.B}$	0.42±0.02 $^{b.B}$
PS-N	0.62±0.05 ^{a,A}	0.42±0.02 ^{b,A}	0.39±0.02 $^{b.B}$	0.41±0.02 $^{b.B}$
PSL-N	0.67±0.04 ^{a,A}	0.47±0.02 ^{b,A}	0.42±0.02 $^{b.AB}$	0.40±0.01 ^{b,B}
Cohesiveness (ratio)				
Con	0.58±0.01 ^{a,A}	0.33±0.02 ^{b,B}	0.31±0.01 ^{b,A}	0.32±0.01 ^{b,B}
PS	0.56±0.01 ^{a,A}	0.38±0.01 ^{b,A}	0.35±0.02 ^{b,A}	0.36±0.01 ^{b,A}
PSL	0.57±0.02 ^{a,A}	0.34±0.01 ^{b,AB}	0.33±0.01 ^{b,A}	0.34±0.02 ^{b,AB}
PS-N	0.56±0.02 ^{a,A}	0.35±0.01 ^{b,AB}	0.32±0.01 ^{b,A}	0.33±0.01 ^{b,AB}
PSL-N	0.60±0.01 ^{a,A}	0.35±0.01 ^{b,AB}	0.33±0.01 ^{b,A}	0.36±0.01 ^{b,AB}
Chewiness (N mm)				
Con	2.81±0.08 ^{c,B}	6.23±0.34 ^{b,A}	7.19±0.12 ^{a,A}	7.01±0.20 ^{a,A}
PS	2.94±0.06 ^{b,AB}	4.88±0.58 ^{a,B}	5.64±0.26 ^{a,B}	5.86±0.50 ^{a,B}
PSL	2.91±0.04 ^{c,AB}	5.80±0.23 ^{b,AB}	6.64±0.24 ^{a,A}	6.67±0.15 ^{a,AB}
PS-N	2.83±0.05 ^{b,AB}	5.93±0.09 ^{a,AB}	6.49±0.38 ^{a,A}	6.57±0.15 ^{a,AB}
PSL-N	3.02±0.06 ^{c,A}	5.59±0.11 ^{b,AB}	6.90±0.18 ^{a,A}	6.62±0.13 ^{a,AB}

$^{a–c}$ Means within the same row with different superscripts are significantly different ($P < 0.05$).

$^{A–C}$ Means within the same column with different superscripts are significantly different ($P < 0.05$).

Con, PS, PSL, PS-N, and PSL-N: abbreviations are the same as Table 11.

Table 15 Changes in texture profiles of mum sausages with/without starter cultures and nitrite during storage (Continuous)

Treatment	Storage time (day)			
	0	3	14	28
Adhesiveness (Ns)				
Con	-0.58±0.02 ^{d,A}	-0.31±0.01 ^{c,A}	-0.26±0.01 ^{b,A}	-0.22±0.02 ^{a,A}
PS	-0.61±0.01 ^{d,A}	-0.47±0.02 ^{c,C}	-0.38±0.01 ^{b,D}	-0.32±0.01 ^{a,D}
PSL	-0.55±0.03 ^{c,A}	-0.39±0.03 ^{b,B}	-0.36±0.02 ^{ab,C}	-0.30±0.01 ^{a,CD}
PS-N	-0.60±0.01 ^{d,A}	-0.45±0.01 ^{c,C}	-0.35±0.01 ^{b,C}	-0.29±0.02 ^{a,BC}
PSL-N	-0.56±0.02 ^{c,A}	-0.41±0.02 ^{b,B}	-0.31±0.03 ^{a,B}	-0.27±0.01 ^{a,B}
Gel strength (J)				
Con	714±46 ^{b,A}	3774±127 ^{a,A}	3917±98 ^{a,A}	3981±87 ^{a,A}
PS	725±61 ^{b,A}	3582±85 ^{a,A}	3725±57 ^{a,AB}	3789±45 ^{a,AB}
PSL	721±66 ^{b,A}	3462±158 ^{a,A}	3606±129 ^{a,AB}	3669±118 ^{a,AB}
PS-N	698±52 ^{b,A}	3597±53 ^{a,A}	3740±24 ^{a,AB}	3804±13 ^{a,AB}
PSL-N	697±43 ^{c,A}	3515±92 ^{b,A}	3658±64 ^{a,B}	3722±52 ^{a,B}

$^{a–d}$ Means within the same row with different superscripts are significantly different ($P < 0.05$).

$^{A–D}$ Means within the same column with different superscripts are significantly different ($P < 0.05$).

Con, PS, PSL, PS-N, and PSL-N: abbreviations are the same as Table 11.

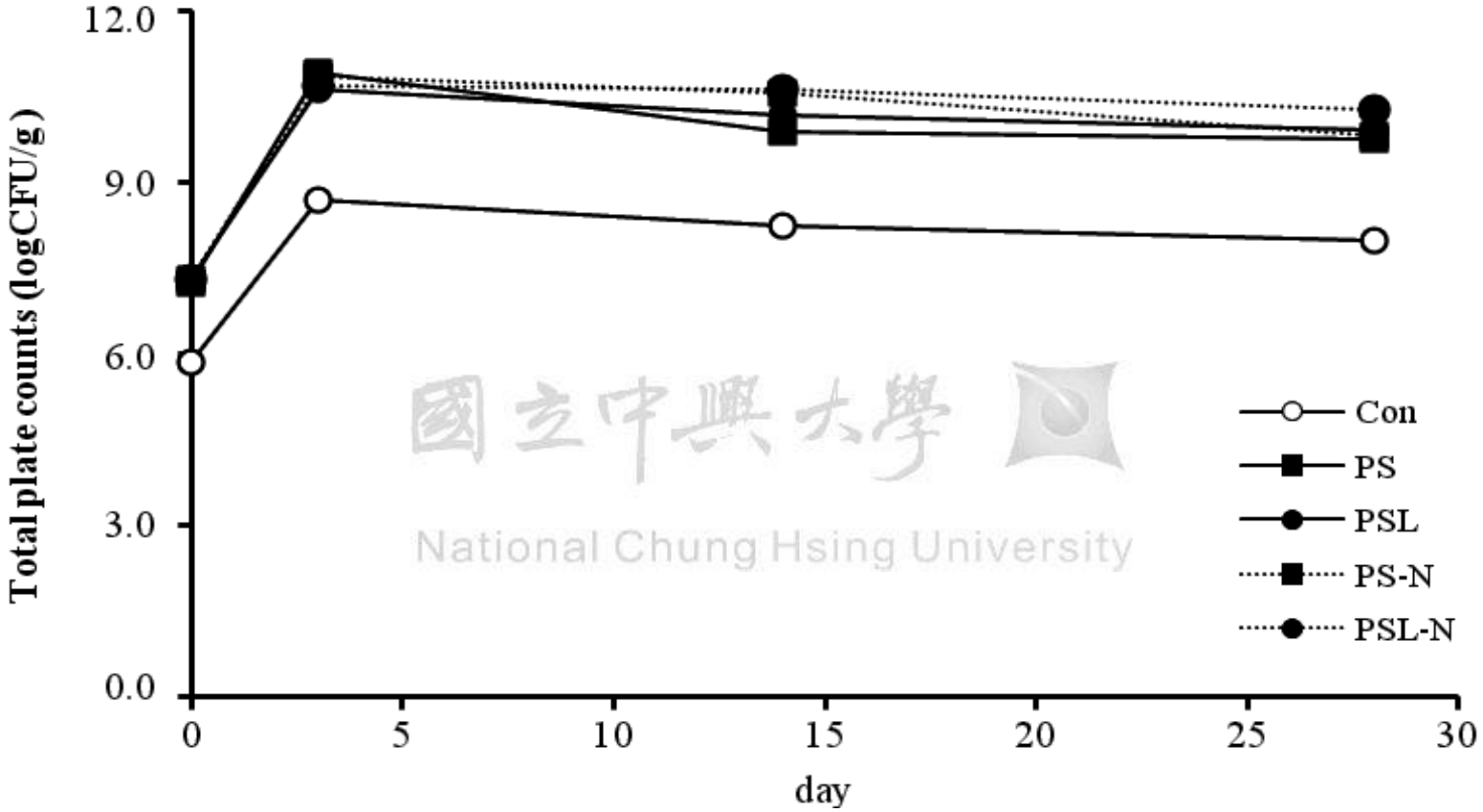


Figure 15. Changes in the total microflora counts of mum sausages with/without starter cultures and nitrite during storage. Abbreviations are the same as Table 11.

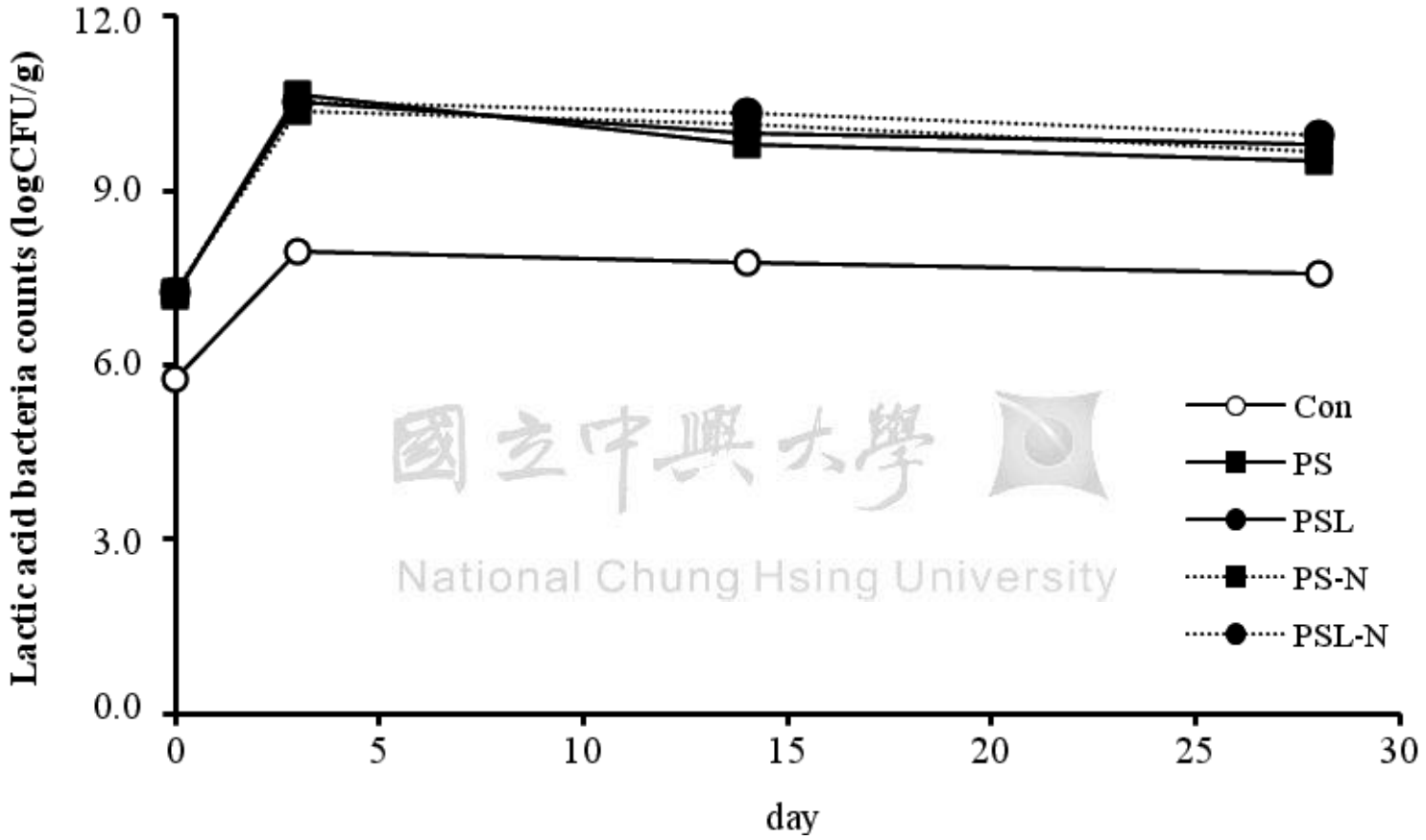


Figure 16. Changes in the lactic acid bacteria counts of mum sausages with/without starter cultures and nitrite during storage. Abbreviations are the same as Table 11.

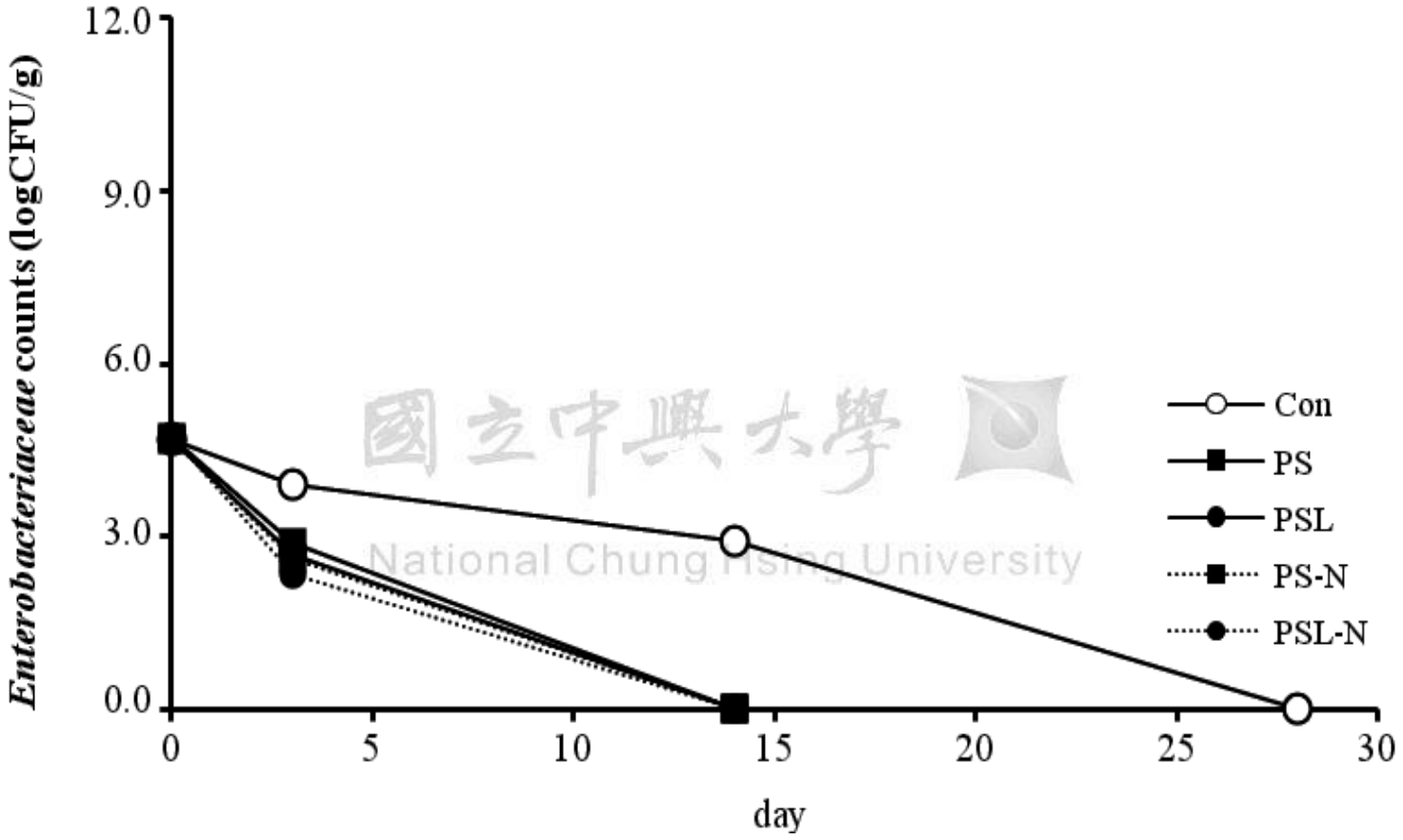


Figure 17. Changes in the *Enterobacteriaceace* counts of mum sausages with/without starter cultures and nitrite during storage. Abbreviations are the same as Table 11.

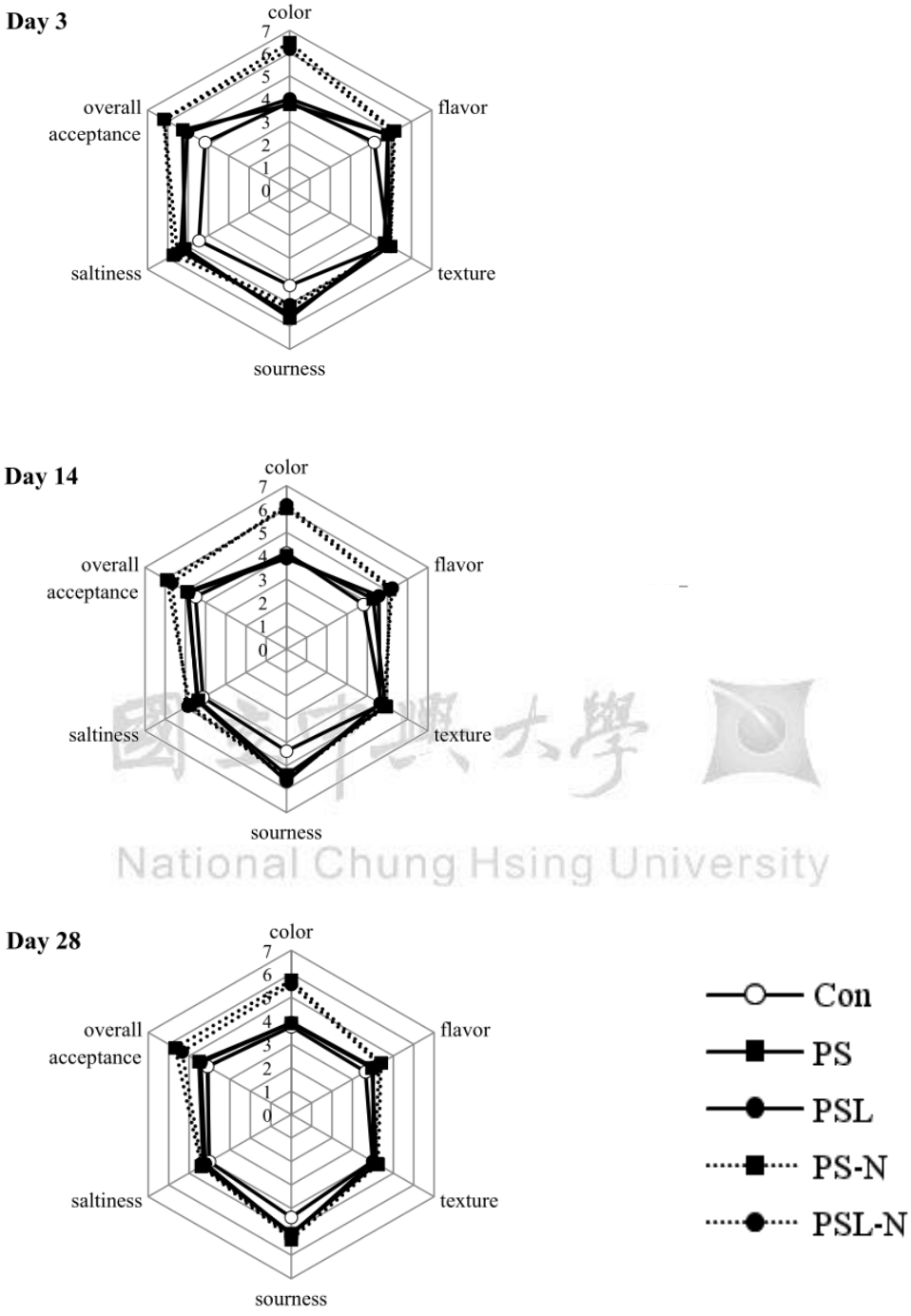


Figure 18. Sensory evaluation of mum sausages of mum sausages with/without starter cultures and nitrite during storage (a) at day 3 (end-fermentation) and (b) at day 14 (storage process) and at day 28 (storage process). Abbreviations are the same as Table 11.

CHAPTER 6

Conclusion

The present study consists of three experiments: Experiment I: An innovative method for the mum preparation with acceptable technological quality and extended shelf-life; Experiment II: Monitoring the dynamics of lactic acid bacteria population during manufacturing and storage of mum based on restriction fragment length polymorphism (RFLP) analysis; Experiment III: The addition of starter cultures and nitrite to improve safety and quality of mum. The results obtained in each part were summarized as follows:

In experiment I, the mum sausages manufactured by the traditional processing method (process I, fermented and stored at ~30°C until day 14) had lower moisture content, which resulted in tough textures, and could not be consumed after 14 days of storage. Contrarily, samples produced using an innovative method (process II, fermented at ~30°C until day 3, vacuum-packaged, and then stored at 4°C until day 28) had more consistent and better qualities. For qualities and shelf-life concerns, mum sausages should be manufactured by this new method. Further studies regarding the microbial and flavor profiles and safety of sausages when applying this innovative method should be also addressed.

In experiment II, the PCR-RFLP assay established in this study showed a rapid and easy differentiation and identification of LAB, which was isolated from the mum sausages during manufacturing and storage. The *Dde* I restriction enzyme provided the highest discrimination capacity. The results obtained from this study defined the distribution and succession of the predominant LAB species of sausages. *L. sakei* and *L. plantarum* were predominant during fermentation whereas *Ln. mesenteroides* increased remarkably during storage. This provided useful requisite information for further development of desirable LAB starters in order to establish a more controllable processing and to give a product with more consistent and better quality. A suitable mum starter culture containing a mix of all or some of the above predominant LAB species now requires consideration.

In experiment III, The addition of mixed starter cultures (*L. plantarum*, *L. sakei* and *Lc. lactis*) with sodium nitrite (125 ppm) was found to be superior in physicochemical (lower in TBARS, VBN, nitrite content and higher red color), microbiological (rapid decreased in *Enterobacteriacea*e), and sensory attributes (high scores for color, flavor and overall acceptance) of final products. This study clearly revealed that mixed starter culture

and additive (nitrite) should be used together to limit the formation of unsafe compounds in traditional sausage and to preserve their original and specific sensory quality. Furthermore, the next step in this research should evaluate the effect of mixed starter cultures and sodium nitrite addition on biogenic amine formation during the manufacture of mum sausage.

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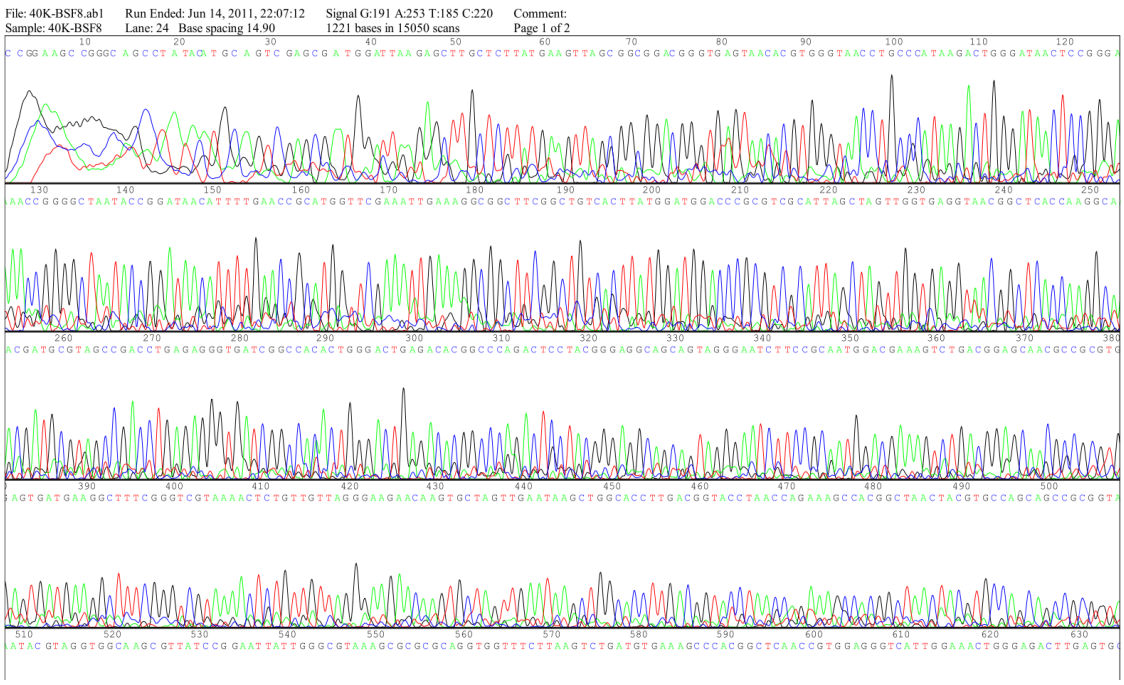
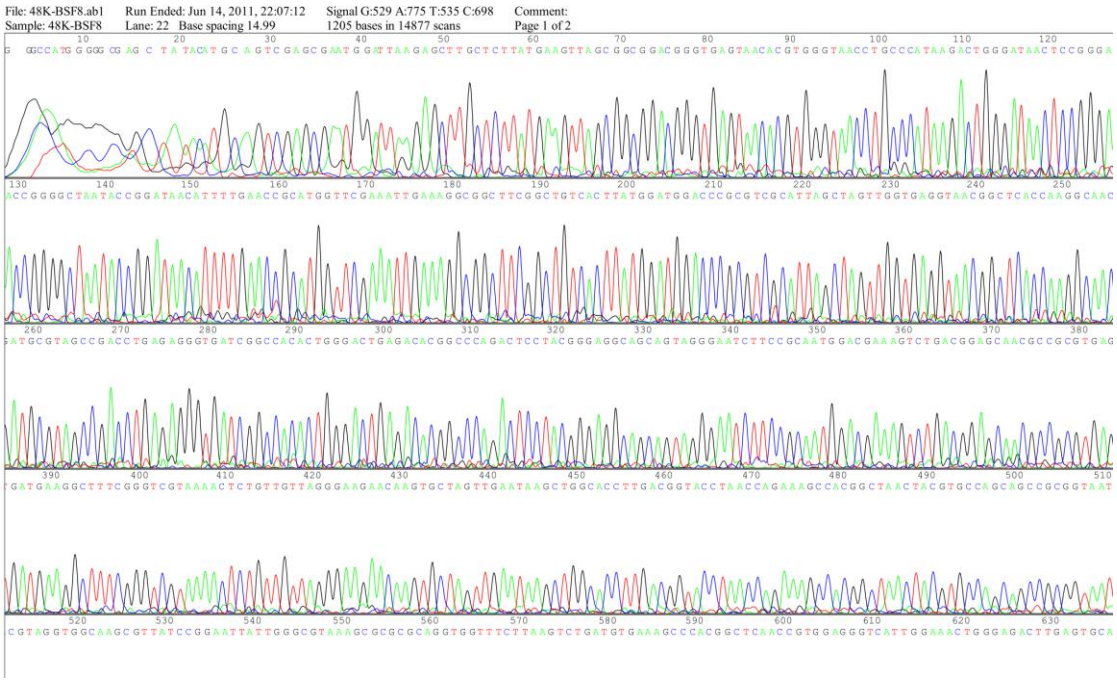
APPENDICES

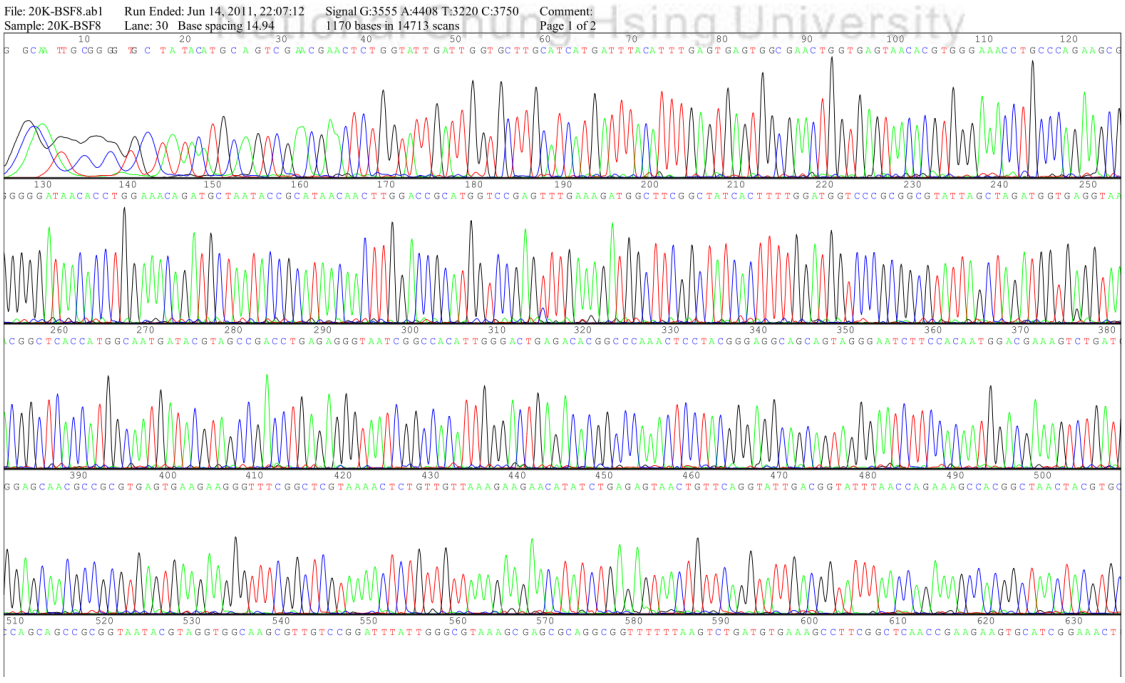


Appendix 1. 16S rDNA sequencing result of RFLP pattern group A in Table 10.

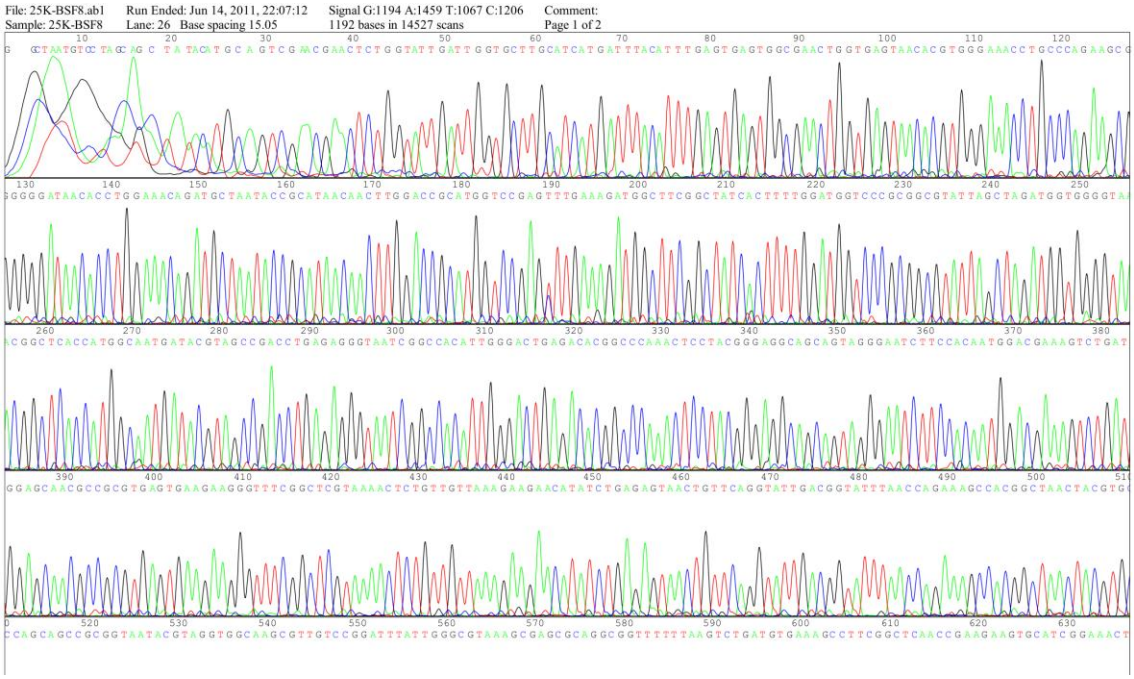
Appendix 2. 16S rDNA sequencing result of RFLP pattern group B in Table 10.



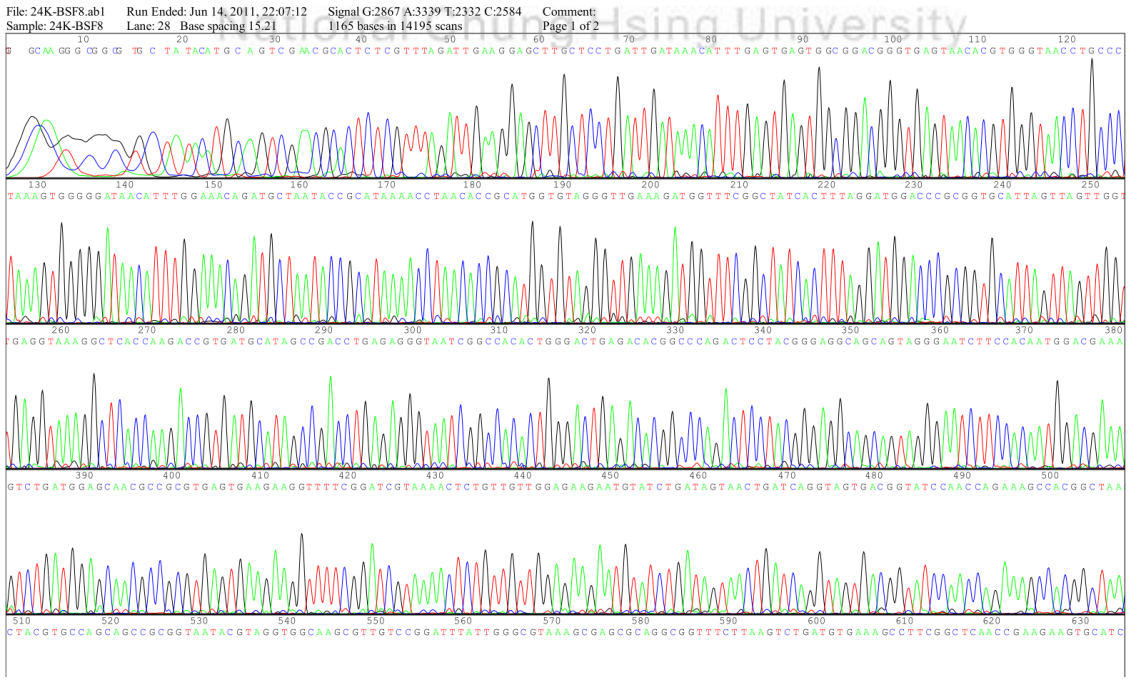
Appendix 3. 16S rDNA sequencing result of RFLP pattern group C in Table 10.



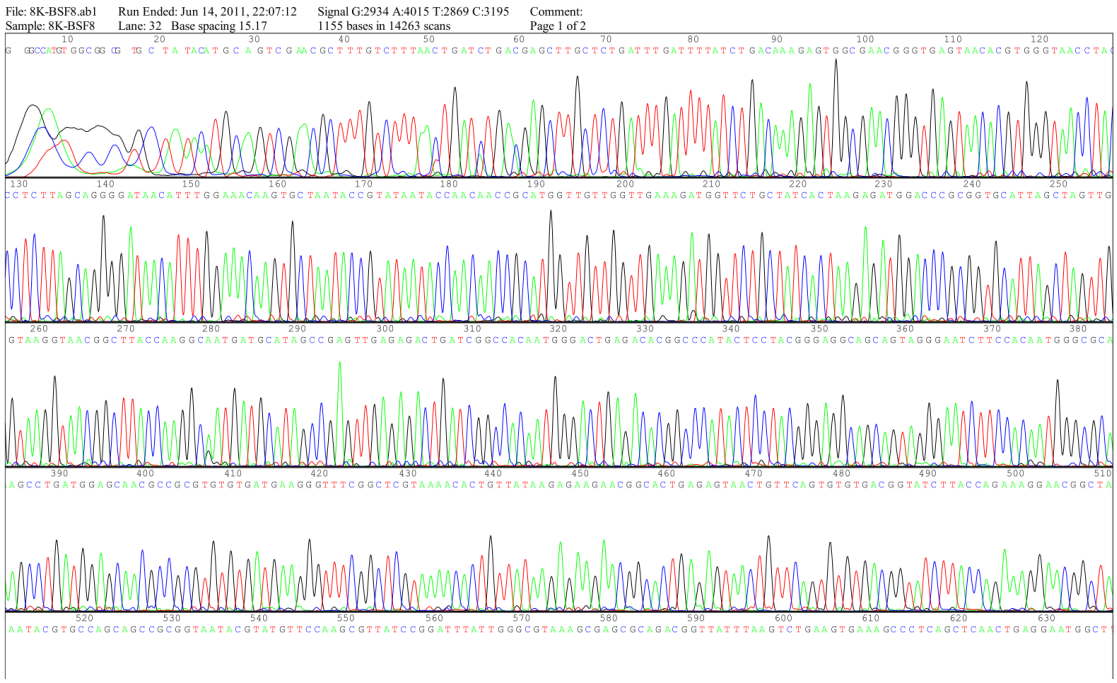
Appendix 4. 16S rDNA sequencing result of RFLP pattern group D in Table 10.



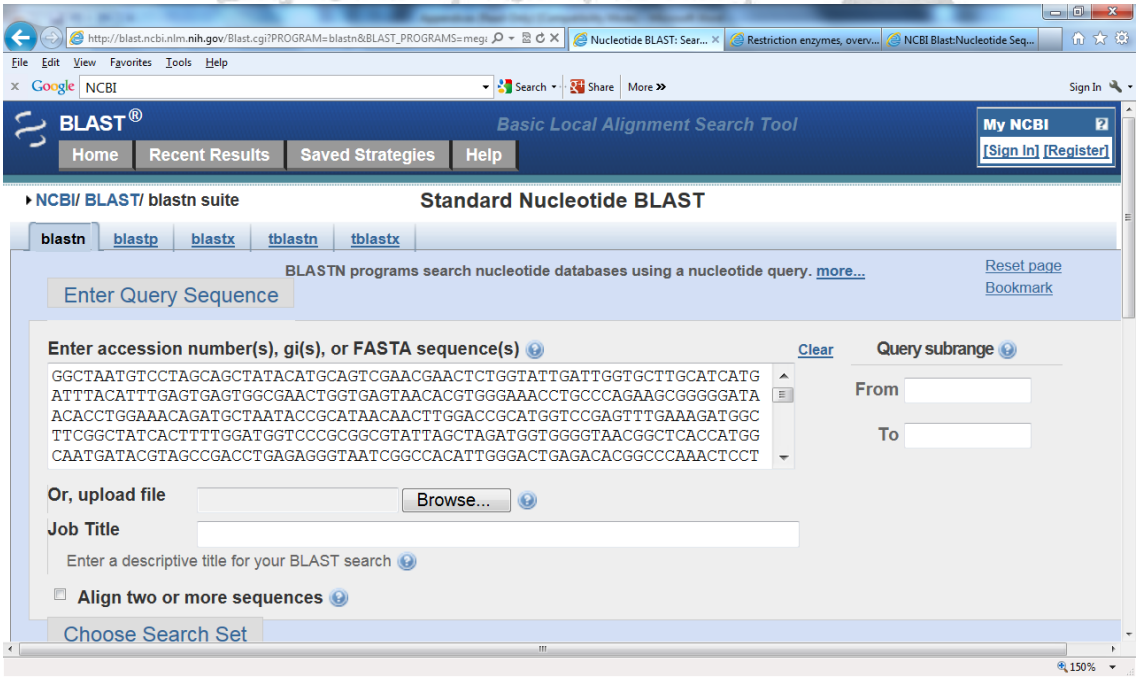
Appendix 5. 16S rDNA sequencing result of RFLP pattern group E in Table 10.



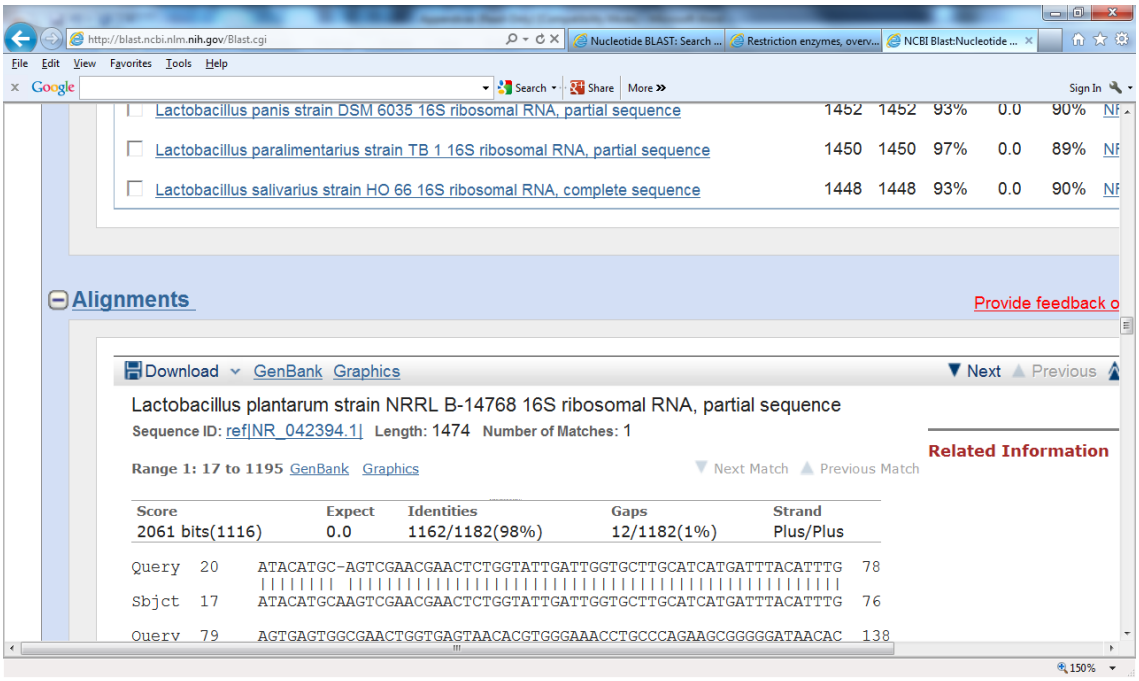
Appendix 6. 16S rDNA sequencing result of RFLP pattern group F in Table 10.



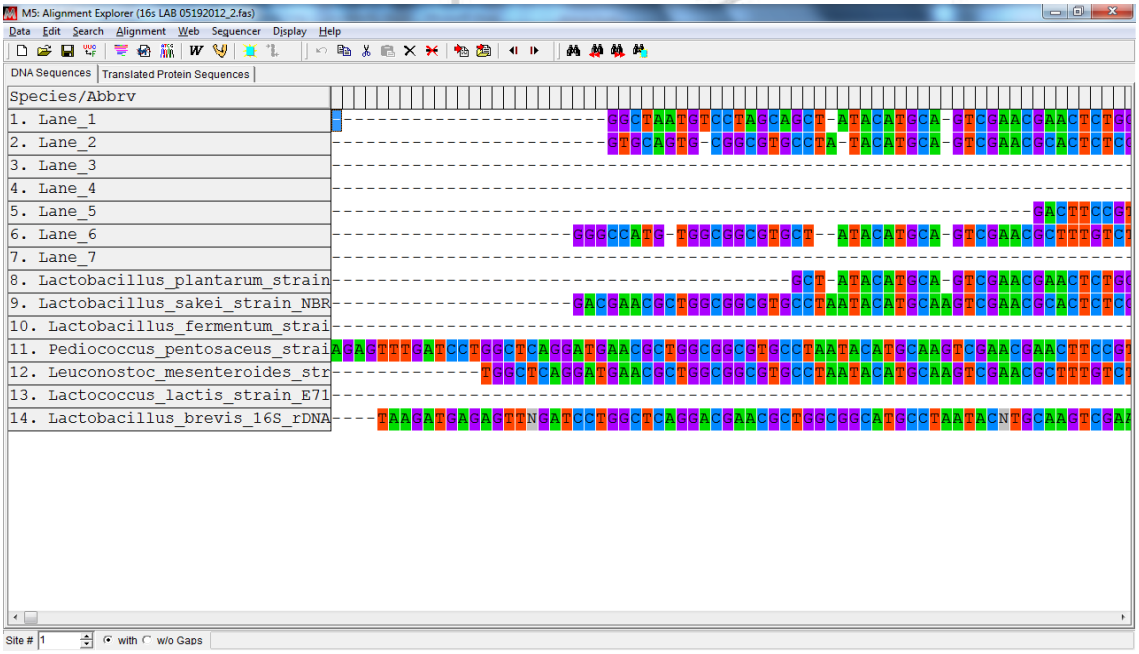
Appendix 7. 16S rDNA sequencing result of RFLP pattern group G in Table 10.



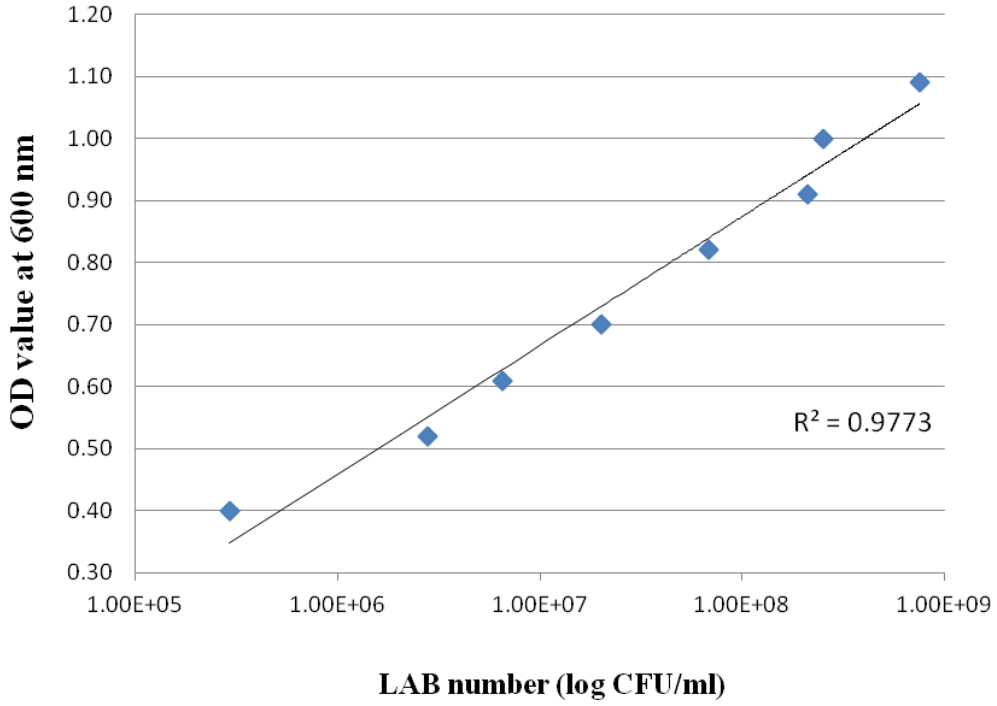
Appendix 8. LAB isolates were identified through a search of the GenBank DNA database (http://www.ncbi.nlm.nih.gov/BLAST).



Appendix 9. GenBank accession numbers of the sequences obtained from the GenBank DNA database (http://www.ncbi.nlm.nih.gov/BLAST).



Appendix 10. Phylogenetic and molecular evolutionary analyses were conducted with the MEGA software



Appendix 11. The standard curve analysis that defines the relationship between an optical density value at 600 nm and lactic acid bacteria number.